F ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

REID, G., Adler
Morgan, Lewis & Bockius LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)
19 December 2000 (19.12.00)

Applicant's or agent's file reference
44574-5044-WO

IMPORTANT NOTIFICATION

International application No.
PCT/US99/27368

International filing date (day/month/year)
19 November 1999 (19.11.99)

1. The following indications appeared on record concerning:

[X] the applicant [X] the inventor [ ] the agent [ ] the common representative

Name and Address
WAXMAN, Stephen, G.
2095 Chapel Street
New Haven, CT 06515
United States of America

State of Nationality: US
State of Residence: US
Telephone No.:
Facsimile No.:
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[ ] the person [ ] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
WAXMAN, Stephen, G.
333 Cedar Street, LCI 707
New Haven, CT 06510
United States of America

State of Nationality: US
State of Residence: US
Telephone No.:
Facsimile No.:
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

Authorized officer
Philippe Bécamel
Telephone No.: (41-22) 338.83.38

PCT/US99/27368

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Assistant Commissioner for Patents
United States Patent and Trademark Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 August 2000 (03.08.00)	
International application No. PCT/US99/27368	**Applicant's or agent's file reference** 44574-5044-WO
International filing date (day/month/year) 19 November 1999 (19.11.99)	**Priority date** (day/month/year) 20 November 1998 (20.11.98)
Applicant DIB-HAJJ, Sulayman, D. et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 June 2000 (20.06.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. Forax
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

WO 00/30670
PCT/US99/27368

EEU
MST
RJP
FF

I ENT COOPERATION TREA

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
REID, G., Adler
Morgan, Lewis & Bockius LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 02 June 2000 (02.06.00)	
Applicant's or agent's file reference 44574-5044-WO	**IMPORTANT NOTICE**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/27368	19 November 1999 (19.11.99)	20 November 1998 (20.11.98)

Applicant
YALE UNIVERSITY et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,JP,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CA,EP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   02 June 2000 (02.06.00) under No. WO 00/30670

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

Th International Bureau of WIPO **34, ch min des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/308 (July 1996)

3309978

PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

To:

REID, G., Adler
Morgan, Lewis & Bockius LLP
1800 M Street, NW
Washington, DC 20036
ETATS-UNIS D'AMERIQUE

INFORMATION CONCERNING ELECTED OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

Date of mailing (day/month/year) 03 August 2000 (03.08.00)	
Applicant's or agent's file reference 44574-5044-WO	**IMPORTANT INFORMATION**

International application No.	**International filing date** (day/month/year)	**Priority date** (day/month/year)
PCT/US99/27368	19 November 1999 (19.11.99)	20 November 1998 (20.11.98)

Applicant
YALE UNIVERSITY et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

   EP :AT,BE,CH,CY,DE,DK,ES,FI,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
   National :AU,CA,JP,US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

   None

3. The applicant is reminded that he must enter the "national phase" **before the expiration of 30 months from the priority date** before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing , if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

   Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

   The entry into the European regional phase is postponed **until 31 months from the priority date** for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer:
R. Forax

Telephone No. (41-22) 338.83.38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BRS, STN, MEDLINE

search terms: GDNF, growth factor, sodium channel, dorsal root ganglia

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-2, drawn to a method to treat pain by administering GDNF to alter Na current flow.
Group II, claim(s) 3-5, drawn to a method to treat pain by administering GDNF to restore Na channels.
Group III, claim(s) 6-8, drawn to a method to treat pain by administering an agent to modulate transcription and translation of mRNA.
Group IV, claim(s) 9-13, drawn to a method to treat pain by administering an agent to modulate GDNF.
Group V, claim(s) 14, drawn to a cell.
Group VI, claim(s) 15-19, drawn to a method to screen candidate compound.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features because the method of claim 1 is anticipated by WILLIAMS (US 5,731,284 A (WILLIAMS) 24 March 1998) and thus, does not share a special technical feature with any other group.

The methods of Groups II-IV and VI, do not share the same or corresponding special technical feature with Group I, because the methods have materially different process steps and are practiced for materially different purposes, and each defines a separate invention over the art.

The product of Group V does not share the same or corresponding special technical feature with Group I, because the product of Group V can be used in a materially different process of protein purification.

Since Groups I-VI do not share a special technical feature, unity of invention is lacking.

Michael Tuscan
202-739-5820

Form PCT/ISA/210 (extra sheet)(July 1992) ★

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 38/00, 38/16, 38/17, 38/18, 38/22

A1

(11) International Publication Number: **WO 00/30670**

(43) International Publication Date: 2 June 2000 (02.06.00)

(21) International Application Number: PCT/US99/27368

(22) International Filing Date: 19 November 1999 (19.11.99)

(30) Priority Data:
60/109,666 20 November 1998 (20.11.98) US

(71) Applicant *(for all designated States except US)*: YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06520 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DIB–HAJJ, Sulayman, D. [US/US]; 110 Lovers Lane, East Lyme, CT 06333 (US). FJELL, Jenny [SE/US]; 265 College Street, Apartment 11J, New Haven, CT 06510 (US). BLACK, Joel, A. [US/US]; 208 Dorrance Street, Hamden, CT 06518 (US). WAXMAN, Stephen, G. [US/US]; 2095 Chapel Street, New Haven, CT 06515 (US). CUMMINS, Theodore, R. [US/US]; 61 Catherine Street, East Haven, CT 06512 (US).

(74) Agent: REID, G., Adler; Morgan, Lewis & Bockius LLP, 1800 M Street, NW, Washington, DC 20036 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

(57) Abstract

The present invention provides a new means for altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes methods to treat pain or hyperexcitability phenomena by altering or modulating the sodium channel expression or activity. The present invention also includes methods of identifying agents which modulate TTX–R Na^+ current through channels, particularly NaN channels, by altering or modulating the level or activity of the channel or by altering or modulating the expression or activity of a neurotrophin.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/00, 38/16, 38/17, 38/18, 38/22
US CL :514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,731,284 A (WILLIAMS) 24 March 1998(24.03.98), columns 16-19.	1-2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 MARCH 2000	Date of mailing of the international search report 18APR2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL PAK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BRS, STN, MEDLINE

search terms: GDNF, growth factor, sodium channel, dorsal root ganglia

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
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The product of Group V does not share the same or corresponding special technical feature with Group I, because the product of Group V can be used in a materially different process of protein purification.

Since Groups I-VI do not share a special technical feature, unity of invention is lacking.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :		(11) International Publication Number:
A61K 38/00, 38/16, 38/17, 38/18, 38/22	A1	WO 00/30670
		(43) International Publication Date: 2 June 2000 (02.06.00)

(21) International Application Number: PCT/US99/27368

(22) International Filing Date: 19 November 1999 (19.11.99)

(30) Priority Data:
60/109,666 20 November 1998 (20.11.98) US

20 May 01/30 mos

(71) Applicant *(for all designated States except US)*: YALE UNIVERSITY [US/US]; 451 College Street, New Haven, CT 06520 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DIB-HAJJ, Sulayman, D. [US/US]; 110 Lovers Lane, East Lyme, CT 06333 (US). FJELL, Jenny [SE/US]; 265 College Street, Apartment 11J, New Haven, CT 06510 (US). BLACK, Joel, A. [US/US]; 208 Dorrance Street, Hamden, CT 06518 (US). WAXMAN, Stephen, G. [US/US]; 2095 Chapel Street, New Haven, CT 06515 (US). CUMMINS, Theodore, R. [US/US]; 61 Catherine Street, East Haven, CT 06512 (US).

(74) Agent: REID, G., Adler; Morgan, Lewis & Bockius LLP, 1800 M Street, NW, Washington, DC 20036 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

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(57) Abstract

The present invention provides a new means for altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes methods to treat pain or hyperexcitability phenomena by altering or modulating the sodium channel expression or activity. The present invention also includes methods of identifying agents which modulate TTX-R Na^+ current through channels, particularly NaN channels, by altering or modulating the level or activity of the channel or by altering or modulating the expression or activity of a neurotrophin.

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EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 09/354,147, filed July 16, 1999, which is a continuation-in-part of PCT International Application No. PCT/US99/02008, also entitled "Modulation of Sodium Channels of Dorsal Root Ganglia," filed January 29, 1999, each of which are related to U.S. Provisional Application 60/072,990, filed January 29, 1998, U.S. Provisional Application 60/109,402 entitled "Modulation of Sodium Channels in Dorsal Root Ganglia", filed November 20, 1998 and to U.S. Provision Application 60/109,666, entitled "Differential Role of GDNF and NGF in the Maintenance of Two TTX-Resistant Sodium Channels in Adult DRG Neurons," filed on November 20, 1998, all of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to the role of glial-derived nerve factor (GDNF) and Nerve Growth Factor (NGF) in modulating the activity of tetrodotoxin (TTX)-resistant sodium channels in dorsal root ganglion (DRG) neurons.

BACKGROUND

Small dorsal root ganglion (DRG) neurons give rise to c- and Aδ-fibers and are predominantly nociceptive (Lynn and Carpenter, 1982; Kress et al., 1992). Many of these neurons display somatic sodium current components that are relatively resistant to tetrodotoxin (TTX) (Kostyuk et al., 1982; Roy and Narahashi, 1992), and it has been suggested that TTX-resistant (TTX-R) sodium currents play an important role in nociceptive transmission (Jeftinija, 1994; Gold et al., 1996). Transcripts for two TTX-R sodium channels, SNS/PN3 and NaN, are preferentially expressed in small DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al., 1998b), and may be

responsible for the TTX-R sodium current observed in the soma (Kostyuk et al., 1981; Bossou and Feltz, 1984; McLean et al., 1988; Caffrey et al., 1992; Roy and Narahashi, 1992) and c-fibers (Quasthoff et al., 1995) of these neurons.

Following transection of the sciatic nerve, TTX-R sodium currents in DRG neurons are attenuated (Rizzo et al., 1995; Cummins and Waxman, 1997) and concomitantly SNS/PN3 (Dib-Hajj et al., 1996; Okuse et al., 1997) and NaN (Dib-Hajj et al., 1998b) transcripts are down-regulated. Axotomy prevents the retrograde transport of nerve growth factor (NGF) from peripheral targets and this may account for many of the phenotypic changes that appear in DRG neurons following axotomy (for review see Verge et al., 1996). In agreement with a role for NGF in maintaining TTX-R sodium currents, infusion of NGF to the transected nerve stump restores SNS/PN3 mRNA to near-normal levels, but only partially rescues TTX-R currents (Dib-Hajj et al., 1998a).

Several mechanisms may explain why NGF only partially restores TTX-R currents following axotomy; an intriguing possibility, however, is that neurotrophins other than NGF regulate the expression of TTX-R sodium channels in the subpopulation of DRG neurons that lack receptors for NGF. Glial cell line-derived neurotrophic factor (GDNF) has been suggested to be important for the maintenance of phenotypic properties in the subset of small sensory neurons that lack NGF receptors (Molliver et al., 1997; Bennett et al., 1998b), and intrathecal administration of GDNF can ameliorate the reduction in conduction velocity in small-diameter axons after sciatic nerve transection (Bennett et al., 1998b). Although a substantial body of evidence demonstrates a role for NGF in the regulation of specific sodium (Kalman et al., 1990; D'Arcangelo et al., 1993; Zur et al., 1995) and potassium (Sharma et al., 1993; Lesser and Lo, 1995) channel expression, the actions of GDNF on ion channel expression has not been established.

GDNF-sensitive and NGF-sensitive neurons can be differentiated by their different ability to bind the lectin IB4 from *Griffonia simplicifolia*. IB4-binding ($IB4^+$) neurons express the receptor/transducing elements necessary to respond to GDNF whereas $IB4^-$ neurons generally express the NGF receptors, TrkA and p75 (Averill et al., 1995; Wright and Snider, 1995; Bennett et al., 1996; Molliver et al., 1997; Bennett et al., 1998b). Based on differences in terminal fields in the spinal cord and certain differences in phenotype of $IB4^+$ and $IB4^-$ neurons, it has been suggested that these two subpopulations of small DRG

neurons may play distinct roles in nociceptive transmission (see Snider and McMahon, 1998).

In the present study, the expression of SNS/PN3 and NaN in $IB4^+$ and $IB4^-$ DRG neurons was examined to determine 1) whether subpopulations of small DRG neurons express distinct TTX-R sodium channels, with different current characteristics and 2) whether NGF and GDNF have differential effects on the expression of SNS/PN3 and NaN, and on TTX-R currents. Inappropriate electrical activity may be involved in some pain syndromes (Matzner and Devor, 19940, and the expression and differential regulation of specific sodium channel gene products in selected sensory neurons may have important implications for pharmaceutical management of pain. Consequently, there is a need for compositions that modulate expression of these genes and also for methods to identify and use the such compositions.

SUMMARY OF THE INVENTION

The present invention provides a new means of altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes a method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF or GDNF-related molecule that is effective to alter TTX-R Na^+ current flow through NaN sodium channels in sensory neurons such as DRG or trigeminal neurons.

The present invention also includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF or a GDNF-related molecule that is capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in sensory neurons such as DRG or trigeminal neurons.

In another embodiment, the invention includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and NaN channels. Such agents includes neurotrophins such as NGF and GDNF.

In another embodiment, the invention includes a method to treat pain, paraesthesia

or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

Another aspect of the invention includes a method of identifying an agent which modulates TTX-R Na^+ current through NaN channels comprising the step of determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

Also, the invention includes a method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na^+ channels.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1. Cell size distribution of DRG neurons positive and negative for SNS/PN3 (A) and NaN mRNA (B). Data for each graph were pooled from four independent experiments by dividing the OD of each neuron by the mean OD of all neurons captured in that experiment. Neurons with a relative intensity > 0.8 are considered positive for SNS/PN3 or NaN. For each size bin, the graphs present the percent of all neurons that are positive and negative for SNS/PN3 (A) or NaN (B). n = 413 for SNS/PN3. n = 263 for NaN. Both SNS/PN3 and NaN are expressed in a large proportion of small DRG neurons.

Figure 2. SNS/PN3 and NaN mRNA in representative $IB4^+$ and $IB4^-$ DRG neurons. IB4 binding, visualized using biotin-labeled IB4 and Cy2-labeled streptavidin, can be readily recognized after *in situ* hybridization. Examples of 1 day *in vitro* ("DIV") neurons processed for *in situ* hybridization and IB4-binding are shown. A,C) IB4 positivity and negativity after SNS/PN3 *in situ* hybridization. B,D) Corresponding Nomarski images of the same neurons. SNS/PN3 mRNA is expressed in both $IB4^+$ (A,B) and $IB4^-$ (C,D) DRG neurons. E,G) IB4 binding after NaN *in situ* hybridization. F,H) Corresponding Nomarski images of the same neurons. NaN mRNA is expressed predominantly in $IB4^+$ neurons (E, F), whereas NaN was undetectable in many $IB4^-$ neurons (G,H). Scale bar is 25 μm.

Figure 3. SNS/PN3 (A) and NaN (B) distribution in small (<30 μm) $IB4^+$ and $IB4^-$

neurons. (A,B) Frequency diagram showing the percentage of $IB4^+$ and $IB4^-$ neurons, respectively, within each hybridization intensity bin (bin-width 0.4 unit). The percentage of neurons is plotted at the midpoint of each bin. The relative intensity was calculated by subtracting the background intensity and then dividing the optical density of each neurons by the mean of all neurons examined in that experiment. Data was pooled from four independent experiments. *A)* SNS/PN3 mRNA is expressed in both in $IB4^+$ and $IB4^-$ DRG neurons. Note that the most intensely stained cells (relative intensity >2.0) were almost exclusively $IB4^-$. The difference in SNS/PN3 hybridization signal between $IB4^+$ and $IB4^-$ neurons was significant ($p< 0.05$, n = 338, Mann-Whitney Rank sum test). *B)* NaN is expressed predominantly in $IB4^+$ DRG neurons. The difference in NaN hybridization signal between $IB4^+$ and $IB4^-$ neurons was significant ($p< 0.001$, n = 242, Mann-Whitney Rank sum test).

Figure 4. Comparison of sodium currents in $IB4^+$ and $IB4^-$ DRG neurons. A) Families of current traces recorded from representative neurons without TTX in the bath are shown. While 97% of the $IB4^+$ neurons exhibited both fast and slow currents (left panel), only 63% of $IB4^-$ neurons exhibited both fast and slow currents (middle panel). 37% of $IB4^-$ neurons exhibited predominantly fast currents (left panel). The currents were elicited by 20 ms test pulses to -10 mV after 500 ms prepulses to potentials over the range of -130 mV to -10 mV. The inset graph in each panel shows the corresponding steady-state inactivation curves for each cell. Current is plotted as a fraction of peak current. Two current components can be easily resolved in the left and middle panels; a slowly inactivating component that has a relatively depolarized voltage-dependence of inactivation (V_h) and a fast inactivating component that has a more negative V_h. The steady-state inactivation curves for these cells are bimodal because of the different inactivation properties of the two components (arrows indicate point of inflection). The $IB4^-$ cell in the right panel, on the other hand, appears to exhibit only fast-inactivating currents and the steady-state inactivation is not inflected. B) The midpoints of steady-state inactivation for the slow current component in $IB4^+$ (solid squares, n=33) and $IB4^-$ F/S (open circles, n=20) neurons are plotted as a function of the slow current density. The fast currents were eliminated using prepulse inactivation. The horizontal dashed lines indicate the average midpoint of inactivation for the slow currents in $IB4^+$ neurons (-37.7±1.0 mV)

and IB4$^-$ F/S neurons (-31.3±1.1 mV). C) Normalized activation (circles) and steady-state inactivation (squares) curves for the slow currents in IB4$^+$ (filled symbols, n=33), and IB4$^-$ (open symbols, n=20) neurons are shown. Cells were held at -100 mV, and prepulsed to -50 mV to inactivate TTX-S currents. Activation was measured with 40 ms test pulses ranging from -70 to +40 mV in 5 mV steps and the midpoint of activation was -23.6±1.3 mV for IB4$^+$ neurons and -17.4±1.6 mV for IB4$^-$ neurons. Steady-state inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. Error bars indicate standard error.

Figure 5. Comparison of TTX-R currents in IB4$^+$ and IB4$^-$ neurons. A) Families of voltage-activated TTX-R current traces recorded from representative IB4$^+$ and IB4$^-$ neurons with 250 nM TTX in the bath are shown. While all of the IB4$^+$ neurons exhibited large (>3 nA) TTX-R currents (left panel; n=30), 60% of the IB4$^-$ exhibited large TTX-R currents (middle panel; n=18) and 40% of the IB4$^-$ cells exhibited little or no TTX-R current (right panel; n=12). The currents were elicited by 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. B) Normalized activation (circles) and steady-state inactivation (squares) curves for the TTX-R currents in IB4$^+$ (filled symbols, n=30), and IB4$^-$ (open symbols, n=18) neurons are shown. Error bars indicate standard error. Cells were held at -100 mV and activation was measured with 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Steady-state fast inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. C) Scatterplots showing the midpoint of steady-state inactivation against the midpoint of activation for TTX-R currents in IB4$^+$ (filled squares) and IB4$^-$ (open circles) neurons. The boxes delineate the mean ± standard deviation for the IB4$^+$ (solid outline) and IB4$^-$ (dashed outline) data.

Figure 6. Effects of neurotrophins on the expression of SNS/PN3 mRNA. The optical densities of neurons captured from three separate experiments were normalized and pooled. The graph represents the mean normalized OD for IB4$^+$ and IB4$^-$ neurons respectively for each condition. Error bar represents standard error. All treatments produced a significant difference in the respective subpopulations ($p_c < 0.01$; Bonferroni *t* test for multiple comparisons), compared to 7 DIV control neurons. Whereas GDNF was significantly ($p_c <$

0.001) more effective on IB4$^+$ neurons compared to IB4$^-$ neurons, no significant difference (p_c = ns) was observed between IB4$^+$ and IB4$^-$ neurons after NGF treatment.

Figure 7. SNS/PN3 *in situ* hybridization of representative IB4$^+$ and IB4$^-$ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4$^+$ A) or IB4$^-$ (B) neurons. NGF increases the signal in both IB4$^+$ (C) and IB4$^-$ (D) neurons. GDNF increased the hybridization signal predominantly in IB4$^+$ neurons (E), with little effect on IB4$^-$ neurons (F). Scale bar is 25 μm.

Figure 8. The upregulation of SNS/PN3 mRNA by NGF is blocked by K252a. The diagram shows the relative SNS/PN3 signal in neurons treated with NGF alone (50 ng/ml; n= 309), or in combination with K252a at 100nM (n= 151), 200nM (n=248) or 400nM (n=65). Each bar represents the mean of the pooled, normalized optical densities from 2 to 4 independent experiments. Error bars represent standard error. K252a alone does not affect the SNS/PN3 hybridization signal intensity at these concentrations (Control: n= 216; 100nM K252a: n= 156; 400nM K252a: n= 86). *The SNS/PN3 hybridization signal was significantly (p_c< 0.001; Bonferroni *t* test) less intense in neurons treated with both NGF and K252a compared to neurons treated with NGF alone.

Figure 9. Effects of neurotrophins on the expression of NaN mRNA. Data from three different experiments are normalized and pooled. The graph represents the mean normalized OD for IB4$^+$ and IB4$^-$ neurons respectively for each condition. Error bar represents standard error. At 7 DIV the NaN hybridization signal was significantly reduced in IB4$^+$ neurons. GDNF increased the NaN hybridization signal in IB4$^+$ neurons, but had no effect on in IB4$^-$ neurons. NGF did not increase NaN expression in either IB4$^+$ or IB4$^-$ neurons. *Significantly different from 7 DIV control by Bonferroni *t* test for multiple comparisons (p_c < 0.001).

Figure 10. NaN *in situ* hybridization of IB4$^+$ and IB4$^-$ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4$^+$ A) or IB4$^-$ (B) neurons. NGF did not increase the signal in either IB4$^+$ (C) or IB4$^-$ (D) neurons. GDNF increased the hybridization signal markedly in IB4$^+$ neurons (E), but had no effect on IB4$^-$ neurons (F). Scale bar is 25 μm.

Figure 11. GDNF increases TTX-R currents in cultured DRG neurons. A)

Families of voltage-activated TTX-R current traces recorded from representative DRG neurons. Currents from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons are shown. Cells were studied after 7 DIV. The bath solution contained 250 nM TTX. The currents were elicited by 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. B) TTX-R peak current amplitude from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons after 7DIV are shown. For comparison, the TTX-R peak current amplitude measured at 1 DIV is also shown. GDNF treatment significantly increases the size of the TTX-R current compared to the control 7 DIV neurons.

Figure 12. Figure 12A shows families of voltage-activated TTX-R current traces recorded from representative SNS-null neurons after 1 day in vitro (1DIV), untreated SNS-null neurons after 7 days in vitro (1DIV) and GDNF-treated SNS-null neurons after 7 days in vitro (DIV). Figure 12B shows TTX-R peak current amplitude from 1DIV, untreated 7DIV, GDNF-treated 7DIV and NGF-treated 7DIV SNS-null neurons.

Figure 13. Figure 13A shows that GDNF administration attenuates the decrease in current density that results from axotomy. Figure 13B shows that GDNF administration attenuates the decrease in persistent Na^+ currents that results from axotomy.

DETAILED DESCRIPTION

Following axotomy, electrophysiological properties of small dorsal root ganglion (DRG) neurons are markedly altered, with attenuation of TTX-R sodium currents and the appearance of rapidly repriming TTX-S currents. The reduction in TTX-R currents has been attributed to a down-regulation of sodium channels SNS/PN3 and NaN. While infusion of exogenous NGF to the transected nerve restores SNS/PN3 transcripts to near-normal levels in small DRG neurons, TTX-R sodium currents are only partially rescued. Binding of the isolectin IB4 distinguishes between two major subpopulations of small DRG neurons: $IB4^+$ neurons, which express receptors for the GDNF family of neurotrophins, and $IB4^-$ neurons that predominantly express trkA.

The present inventors have shown that SNS/PN3 is expressed in approximately one-half of both $IB4^+$ and $IB4^-$ DRG neurons, while NaN is preferentially expressed in $IB4^+$ neurons. Whole-cell patch-clamp studies demonstrate that TTX-R sodium currents in $IB4^+$ neurons have a more hyperpolarized voltage-dependence of activation and inactivation than do $IB4^-$ neurons, suggesting different electrophysiological properties for SNS/PN3 and NaN. Utilizing an *in vitro* model of axotomy, it was confirmed that NGF restores SNS/PN3 mRNA levels and demonstrate that the trk antagonist K252a blocks this rescue, indicating a central role for trkA receptors in the signaling pathway. The down-regulation of NaN mRNA is, nevertheless, not rescued by NGF-treatment in either $IB4^+$ or $IB4^-$ neurons and NGF-treatment does not significantly increase the peak amplitude of the TTX-R current in small DRG neurons *in vitro*. In contrast, GDNF-treatment causes a two-fold increase in the peak amplitude of TTX-R sodium currents and restores both SNS/PN3 and NaN mRNA to near-normal levels in $IB4^+$ neurons. These observations provide a mechanism for the partial restoration of TTX-R sodium currents by NGF in axotomized DRG neurons, and demonstrate that the neurotrophins NGF and GDNF differentially regulate sodium channels SNS/PN3 and NaN.

A. TTX-R sodium channels in $IB4^+$ and $IB4^-$ DRG neurons

Several groups have suggested that DRG neurons exhibit at least two distinct types of TTX-R currents based on the voltage-dependence of activation and inactivation (Brau and Elliott, 1998; Rush et al., 1998; Scholz et al., 1998). The present inventors' results

indicate that NaN and SNS/PN3 could underlie these distinct TTX-R currents in small DRG neurons. Expression of SNS/PN3 in *Xenopus* oocytes gives rise to voltage-gated sodium currents with slow kinetics and resistance to high concentrations of TTX (Akopian et al., 1996; Sangameswaran et al., 1996). NaN, while not as yet heterologously expressed, is predicted to be TTX-R based on sequence analysis (Dib-Hajj et al., 1998b). Whole-cell patch-clamp recordings of $IB4^+$ and $IB4^-$ neurons revealed that the TTX-R currents show a voltage-dependence of steady-state activation and inactivation that is hyperpolarized in $IB4^+$ neurons. While two-thirds of $IB4^-$ cells express TTX-R currents, only about a third express NaN mRNA. Thus, SNS/PN3 may underlie much of the slow TTX-R current that is observed in $IB4^-$ cells. In support of this speculation, SNS/PN3 channels expressed in *Xenopus* oocytes give rise to slow TTX-R currents with a midpoint of inactivation of -30 mV (Akopian et al., 1996), which is similar to what it was observed in the majority of $IB4^-$ cells. Conversely, although all $IB4^+$ neurons display slow TTX-R currents, only about one half express SNS/PN3 mRNA. Since NaN is expressed predominantly in $IB4^+$ neurons, NaN may account for much of the slow TTX-R current that is observed in $IB4^+$ cells. Based on these observations, it is not unreasonable to conclude that NaN corresponds to a TTX-R sodium channel with a more negative midpoint of inactivation than SNS/PN3 and a lower threshold for activation.

The present inventors' observations that NaN is expressed in about 70% of small $IB4^+$ neurons, and that SNS/PN3 mRNA is present in slightly more than 50% of both $IB4^+$ and $IB4^-$ small neurons, indicates that NaN and SNS/PN3 are coexpressed in some of the cells. It would be predicted, therefore, that some neurons would express ensembles of NaN and SNS/PN3 currents and hence the electrophysiological analysis of $IB4^+$ and $IB4^-$ cells would underestimate the difference between the two channels. Coexpression of two slow TTX-R channels with subtly different voltage dependencies could in part account for the interneuronal variation that has been described for slow TTX-R currents in small DRG neurons (Rizzo et al., 1994). Functionally, nociceptive neurons might fine-tune their integrative and repetitive firing properties by altering the relative expression of SNS/PN3 and NaN channels. These channels may also differ in other properties, such as subcellular localization and sensitivity to second messenger modulation, which could also be important determinants of transductive and/or encoding characteristics of different DRG

neurons.

Most IB4$^+$ neurons expressed slow TTX-R currents that were similar to the TTX-R2 currents described by Rush et al. (1998); on the other hand, the TTX-R1 currents were similar to the predominant TTX-R current in IB4$^-$ cells. Fast TTX-R currents such as those described by Scholz et al. (1998) in either IB4$^+$ or IB4$^-$ cells were not observed. In the IB4$^-$ group, about one-third of the small neurons had very low amplitude, or no TTX-R currents, suggesting that this group of neurons expressed neither SNS/PN3 nor NaN. This subpopulation of IB4$^-$ neurons, which expressed relatively large fast TTX-S currents, may represent a distinct subset of sensory neurons.

B. Effect of neurotrophins on SNS/PN3 and NaN mRNA and TTX-R sodium currents

NGF has previously been shown to play a prominent role in the regulation of sodium channel/current expression in PC12 cells, as well as DRG neurons. In PC12 cells, NGF up-regulates sodium channels II and PN1 through distinct signal transduction pathways, with the latter being Ras-independent (D'Arcangelo et al., 1993). Interestingly, short-term (1-minute) application of NGF up-regulates PN1 but not brain type II in PC12 cells (Toledo-Aral et al., 1995). These observations point to divergent signaling pathways for two distinct TTX-S sodium channels (Noda et al., 1986; Klugbauer et al., 1995). Similar mechanisms may regulate specific sodium channel isoforms in DRG neurons. Consistent with this idea, NGF application accelerates the diversity and acquisition of sodium currents in neonatal DRG neurons (Omri and Meiri, 1990) and increases the threshold for spike generation in young post-natal DRG neurons (Aguayo and White, 1992).

The role of NGF in the regulation of SNS/PN3 mRNA in DRG neurons has been the focus of several studies. Administration of exogenous NGF increases the levels of SNS/PN3 mRNA both *in vitro* and *in vivo* (Black et al., 1997; Dib-Hajj et al., 1998a; this study). Moreover, enhanced levels of tissue NGF in the receptive fields of DRG neurons in carrageenan-induced inflammation are associated with up-regulation of SNS/PN3 (Tanaka et al., 1998), while, in contrast, depleted levels of NGF *in vivo* are accompanied by a down-regulation of SNS/PN3 (Fjell et al., 1999b). These observations point to an

important modulatory role for NGF in SNS/PN3 expression. In contrast, utilizing differing methodological and model systems, Okuse et al. (1997) have provided data suggesting a more limited role for NGF in SNS/PN3 expression. However, in the present study, the present inventors have confirmed the earlier observation that NGF up-regulates SNS/PN3 to near-normal levels in an *in vitro* model of axotomy, and have extended these results to show that the action of NGF on SNS/PN3 expression is not limited solely to $IB4^-$ neurons, but can also be detected in $IB4^+$ neurons. The NGF-induced up-regulation of SNS/PN3 was blocked by the trk antagonist K252a (Kase et al., 1987), suggesting that this modulatory action is mediated through a direct effect on the DRG neurons. Since a subpopulation of $IB4^+$ neurons express trkA (Bennett et al., 1998b), the increase in SNS/PN3 mRNA seen in $IB4^+$ neurons after treatment with NGF may reflect an effect on those neurons that also express trkA. NGF, however, does not regulate the expression of all sodium channel mRNAs in DRG neurons that express TrkA receptors, as NGF had no detectable effect on NaN mRNA expression.

While GDNF has well-established roles as a potent survival factor for certain classes of neurons (Lin et al., 1993; Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) and as a protector of neurons from injury (Tomac et al., 1995; Beck et al., 1995), the effect of this neurotrophin on the electrical properties of neurons is largely unknown. It has been previously shown that intrathecal administration of GDNF ameliorates the reduction in conduction velocity of c-type fibers that follows axotomy (Bennett et al., 1998b), although it is unclear what mechanism is responsible for the enhancement of conduction velocity. The present inventors' work demonstrate here for the first time that GDNF modulates sodium channel expression, upregulating both SNS/PN3 and NaN transcripts. The action of GDNF was substantially more pronounced in $IB4^+$ neurons than in $IB4^-$ neurons. Since almost all $IB4^+$, but few $IB4^-$, neurons express receptors for the GDNF-family of neurotrophins (Bennett et al., 1998b), these findings are consistent with an action of GDNF only on those neurons that express the receptor/transducer complex for GDNF.

NGF and GDNF share several characteristics as target-derived, retrogradely-transported neurotrophic factors. In the adult, NGF is primarily expressed in the skin; the levels of NGF are increased in inflamed tissues, and both endogenous and exogenous

increase of tissue NGF levels are associated with pain/hyperalgesia (Lewin et al., 1994; Woolf, 1996; Woolf et al., 1996; Dyck et al., 1997). Conversely, NGF-deprivation *in vivo* prevents hypersensitivity and results in thermal hypoalgesia (Chudler et al., 1997; Bennett et al., 1998a). In contrast to NGF, GDNF is produced at very low levels in adult skin and spinal cord, but is expressed by Schwann cells in the sciatic nerve (Nosrat et al., 1996; Widenfalk et al., 1997), suggesting that Schwann cells may be the primary source of GDNF for adult DRG neurons. It has been suggested that an up-regulation of GDNF in the injured nerve may play an important role for regeneration of sensory neurons following axotomy (Trupp et al., 1995; Hammarberg et al., 1996; Naveilhan et al., 1997; Bär et al., 1998;). Unlike the pain-inducing effect of NGF, McMahon and coworkers have suggested that GDNF may not induce pain (Bennett et al., 1998b). This hypothesis is intriguing in light of the observations of divergent central projections of $IB4^+$ and $IB4^-$ neurons (Molliver et al., 1995), and the suggestion that the former neurons are critically important in neuropathic pain and the latter in inflammatory pain (see Snider and McMahon 1998). The finding that NGF increases the expression of SNS/PN3, but not NaN mRNA, might suggest that some inflammatory syndromes could arise from an imbalance in sodium channels reflecting different responses to different neurotrophic factors. As noted above, SNS/PN3 may encode a TTX-R sodium current with a more depolarized inactivation curve and a higher threshold for activation. Changes in the levels of NGF and GDNF might therefore affect the electrogenic properties of some small DRG neurons after nerve injury, possibly contributing to the development of hyperexcitibility.

C. Methods of screening for agents to alter or modulate Sodium channel expression or activity.

Several approaches can be used to identify agents that are able to alter or modulate the GDNF induced Na^+ current through the SNS/PN3, NaN or other sodium channels. As used herein, "alter" refers to up- or down-regulating the levels or activity of NaN, such as current flow. In general, to identify such agents, a model cultured cell line that expresses the NaN sodium channel and GDNF or a GDNF receptor is utilized, and one or more conventional assays are used to measure Na^+ current. Such conventional assays include, for example, patch clamp methods, the ratiometric imaging of $[Na^+]_i$, and the use of ^{22}Na

and ^{86}Rb. Alternatively, the amount of NaN RNA or protein may be directly measured by conventional assays such as hybridization of immunoblot assays.

In one embodiment of the present invention, to evaluate the activity of a candidate compound to modulate Na^+ current, an agent is brought into contact with a suitable transformed host cell that expresses a functional GDNF receptor and NaN or GDNF. Cells that express endogenous NaN are also useful for screening of candidate agents. After mixing or appropriate incubation time, the Na^+ current is measured to determine if the agent inhibited or enhanced Na^+ current flow. If the cell line is engineered to express a functional GDNF receptor, the agent to be tested may be brought into contact with a suitable host cell in the presence or absence of exogenously supplied GDNF. Agents that inhibit or enhance Na^+ current are thereby identified.

The preferred agents that alter or modulate the levels or activity of NaN preferably will be selective for the NaN Na^+ channel, may be selective for GDNF or may alter or modulate the GDNF mediated induction of NaN. Similar analyses may be conducted by the skilled artisan to identify agents that alter the effect of other neurotrophic factors. For example, Mildbrandt, J. *et al.*. (Neuron, vol. 20, 245-253) describes the discovery of a third member of the GDNF family, Persephin, and reviews the literature on this family. The other two members are: GDNF (the prototype) and nurturin (NTN). Four receptors for these ligands have been identified. See also the March 29th, 1996 issue of the journal: Philosophical Transactions of the Royal Society of London, B. Biological Sciences (philos. trans. R. Soc. Lond. B. Biol. Sci. 1996) which contains multiple chapters on the NGF family of neurotrophic factors and their role in various models.

Agents of the invention may be totally specific (like tetrodotoxin, TTX, which inhibits sodium channels but does not bind to or directly effect any other channels or receptors), or relatively specific (such as lidocaine which binds to and blocks several types of ion channels, but has a preference for sodium channels). Total specificity is not required for an inhibitor or enhancer to be effective; the ratio of its effect on sodium channels vs. other channels and receptors, will determine its effect; and effects on several channels, in addition to the targeted one, may be of interest.

It is contemplated that modulating agents of the present invention can be, as

examples, peptides, small molecules, naturally occurring or synthetic toxins and vitamin derivatives, as well as carbohydrates. A skilled artisan will readily recognize that there is no limit as to the structural nature of the modulating agents of the present invention. It is contemplated that the screening of libraries of molecules will reveal agents that modulate NaN or current flow through it. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the of the NaN Na^+ channel, of GDNF or a functional GDNF receptor. Such peptide fragments can be routinely identified by exposing a transformed host cell to these agents and measuring any resultant changes in Na^+ current. Similarly, naturally occurring toxins (such as those produced by certain fish, amphibians and invertebrates) can be screened.

D. Methods of treating pain, paraesthesia or hyperexcitability phenomena.

Agents of the invention may be administered to a human or animal subject. As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by the alteration or modulation of sodium channels such as NaN. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, alteration or the modulation of the amount or of a biological activity of NaN may be associated with pain, paraesthesia or hyperexcitability phenomena. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, pain, paraesthesia or hyperexcitability phenomena may be prevented, altered or modulated by the administration of agents which reduce, enhance or modulate in some way GDNF induction of NaN.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention, such as GDNF, a GDNF-related molecule such as a GDNF peptide, or a NaN peptide can be administered in combination with another agent that alters or modulates Na^+ current. As used herein, two agents are said to be administered in

combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 μg/kg body wt. The preferred dosages comprise 0.1 to 10 μg/kg body wt. The most preferred dosages comprise 0.1 to 1 μg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules,

pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

GENERAL METHODS

1. *Cell culture*

Adult female Sprague-Dawley rats were deeply anesthetized with xylazine/ketamine (40/2.5 mg/kg; i.p.) and decapitated. The L4 and L5 ganglia were quickly removed and desheathed in sterile complete saline solution (CSS) (pH 7.2). The DRGs were then enzymatically digested for 20 min with collagenase A (1mg/ml; Boerhinger-Mannheim, Indianapolis, IN) in CSS and for 15 min. with collagenase D (1mg/ml; Boerhinger-Mannheim) containing papain (30 units/ml, Worthington Biochemical Corporation, Lakewood, NJ) in CSS at 37°C. The DRGs were gently centrifuged (100g for 3 min.) and the pellet triturated in DRG-media (DMEM:F12, 10% FCS) containing 1mg/ml bovine-serum albumin (BSA, Fraction V) (Sigma, St. Louis, MO) and 1 mg/ml trypsin inhibitor (Sigma). The cells were then plated on poly-ornithine/ laminin-coated glass coverslips and incubated at 37° C in a humidified 95% air /5% CO_2 incubator.

2. *Growth factors*

To study the effects of GDNF, NGF and brain-derived neurotrophic factor (BDNF) on SNS/PN3 and NaN mRNA expression, and TTX-R sodium currents, cells were treated with DRG media or DRG media supplemented with NGF (50 ng/ml, mouse 7S NGF, Sigma), GDNF (human recombinant, 50 ng/ml, Calbiochem, San Diego, CA), BDNF

(10ng/ml, Regeneron) or DRG media supplemented with a combination of NGF (50 ng/ml) and GDNF (50 ng/ml). The cells were maintained in culture for seven days and one-half of the media was replaced daily. For each experiment a control DRG culture containing neurons derived from L4/L5 ganglia was established and maintained for one day *in vitro* (1 DIV). In some experiments the trk inhibitor K252a was used to determine if the effect of NGF on SNS/PN3 mRNA expression is mediated through the trkA pathway. K252a (Calbiochem) was dissolved in DMSO (1mg/ml) and added to cultures in concentrations ranging from 100 nM - 400 nM.

3. In situ hybridization

The expression of SNS/PN3 and NaN mRNA in individual neurons was determined by *in situ* hybridization as previously described (Black et al., 1996; Dib-Hajj et al., 1998b). In short, coverslips from the different experimental groups were fixed for 10 min. in 4% formaldehyde in 0.14M Sorensons buffer, pH 7.2, washed several times with diethylpyrocarbonate (DEPC) -treated PBS and permeabilized with 0.1 % Triton X-100 in PBS for 15 min. The coverslips were then rinsed with 2X SSC, prehybridized for 30 min and then hybridized at 58°C overnight using riboprobes (0.25-0.5 ng/ml) specific for SNS/PN3 or NaN. The coverslips were sequentially incubated in 4X SSC, 2X SSC, RNase A (20mg/ml; Sigma; 37°C, 30 min.) and finally 0.2X SSC at 58°C for 3 X 20 min. The coverslips were then blocked with 2% normal sheep serum and 1 % BSA for 20 min and incubated with alkaline phosphates-conjugated anti-digoxigenin F'ab fragments (1:500, Boerhinger-Mannheim) overnight at 4°C. Following multiple rinses, the hybridization signal was visualized using NBT histochemistry. Coverslips for each condition in each experiment were kept in the NBT solution for the same length of time; the NBT reaction was monitored visually and stopped before the signal reached saturation.

For co-localization of SNS/PN3 or NaN mRNA with IB4 reactivity, biotin labeled-isolectin B4 (IB4) (40µg/ml, Sigma) was added to the culture medium and incubated for 30 min. at 37°C prior to beginning the *in situ* hybridization protocol. The coverslips were then washed with CSS and *in situ* hybridization was performed as described above with minor modifications. Following hybridization and stringent washes, the coverslips were incubated with streptavidin-Cy2 (40µg/ml, Amersham Life Science Inc, Arlington

Heights, IL) and alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Boerhinger-Mannheim) in Tris-buffered blocking solution (1% BSA, 2% normal goat serum) overnight at 4°C.

4. *Quantification and data analysis*

The coverslips were examined with a BioRad MRC-600 confocal microscope equipped with brightfield and BHS filter or with a Leica Aristoplan microscope. IB4 reactivity was determined visually by the presence of extracellular fluorescent signal above background levels. Microdensitometric quantification of the SNS/PN3 and NaN hybridization signal was performed as previously described (Black et al., 1997). Briefly, optical density (OD) measurements of the neurons were obtained using the Scion image analysis program. The brightfield gray levels were linearly (R^2 >0.99) calibrated to optical density using optical filters with OD = 0.1, 0.3 and 0.6. All hybridization signals measured were within the linear calibration range. Samples for analysis were obtained from each coverslip by arbitrarily scrolling the coverslip from the upper left quadrant and capturing the first twenty to fifty fields containing distinguishable neurons. The neurons in the captured images were outlined and the area and mean optical density of each cell was determined. To permit pooling of data from different experiments the optical densities were normalized by dividing the OD of each neuron by the mean SNS/PN3 or NaN optical density of the control cells at 1 DIV, processed in the same *in situ* hybridization experiment. In experiments that colocalized SNS/PN3 or NaN mRNA with IB4 binding, the OD of each $IB4^+$ or $IB4^-$ neuron was normalized as described above using the mean optical density of all ($IB4^+$ and $IB4^-$) neurons. Neurons were considered positive for SNS/PN3 or NaN if the relative intensity was > 0.8, which corresponds to a lightly stained neuron. In experiments with K252a added to the medium, the mean OD of all neurons from cultures that had not received any supplement was used to normalize the experimental values.

The Mann-Whitney Rank sum test was used to test if statistically significant differences exist in the expression of SNS/PN3 and NaN mRNA in $IB4^+$ and $IB4^-$ neurons. To determine if the effects of the different neurotrophins on SNS/PN3 and NaN mRNA expression in $IB4^+$ and $IB4^-$ neurons were statistically significant, the means of the normalized optical densities pooled from 3 separate experiments were analyzed using a

one-way ANOVA. If a significant difference was detected, a two-tailed Student *t* test was used and the resulting p-value was corrected by multiplying by the number of comparisons made (Bonferroni *t* test). Significance was assessed as $p_{\text{corrected}}$ (p_c) < 0.05.

5. *Whole-cell Recordings*

Sodium currents were recorded from DRG neurons in the whole-cell patch-clamp configuration 18-30 hrs. after dissociation and plating (1DIV) or after treatment with growth factors for seven days *in vitro* (7 DIV). Prior to recording, the cells were incubated for 30-60 minutes with FITC-labeled Isolectin B4 (40μg/ml, Sigma). All recordings were made with an EPC-9 amplifier, a Macintosh Quadra 950 and the Pulse program (v 7.52, HEKA Electronic, Germany). Recording electrodes (0.8-2 MΩ) were fabricated from 1.65-mm capillary glass (WPI) using a Sutter P-87 puller. Cells were not considered for analysis if the initial seal resistance was less than 1 GΩ or if they had high leakage currents (holding current > 1 nA at - 80 mV) or an access resistance greater than 5 MΩ. The average access resistance was 2.3 ± 0.8 MΩ (mean ± standard deviation, n=310). Voltage errors were minimized using 70-80% series resistance compensation. Linear leak subtraction and capacitance artifact cancellation were used for all recordings. Membrane currents were filtered at 2.5 KHz and sampled at 10 KHz. The pipette solution contained (in mM): 140 CsF, 2 $MgCl_2$, 1 EGTA, and 10 Na-HEPES (pH 7.3). The standard extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 0.1 $CdCl_2$, and 10 HEPES (pH 7.3). Cadmium was included to block calcium currents. The osmolarity of the solutions was adjusted to 310 mosM (Wescor 5550 osmometer). The liquid junction potential for these solutions was <7 mV; data were not corrected to account for this offset. The offset potential was zeroed before patching the cells and checked after each recording for drift. All recordings were conducted at room temperature (~22° C).

Example 1: ***SNS/PN3 and NaN mRNA in IB4$^+$ and IB4$^-$ DRG neurons***

Both SNS/PN3 and NaN mRNA are suggested to encode TTX-R sodium channels in DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al.,

1998b), and both are preferentially expressed in small (< 30 μm) DRG neurons (Fig. 1). Small DRG neurons have been differentiated into two major subpopulations on the basis of their ability to bind to the lectin IB4 (Averill et al., 1995; Wright and Snider, 1995; Molliver et al., 1997; Bennett et al., 1998b). To establish if SNS/PN3 and NaN are differentially expressed in $IB4^+$ and $IB4^-$ neurons, localization of SNS/PN3 or NaN mRNA by *in situ* hybridization was combined with IB4 cytofluorescent labeling. IB4 binding was determined by the presence of a clearly identifiable extracellullar staining of the soma (Fig. 2). SNS/PN3 mRNA was expressed in both $IB4^+$ and $IB4^-$ neurons, but neurons with the greatest hybridization signal for SNS/PN3 mRNA were predominantly $IB4^-$. The difference in SNS/PN3 mRNA expression between $IB4^+$ and $IB4^-$ neurons was significant ($p < 0.05$, Mann-Whitney rank sum test) (Fig. 3a). Fifty-two percent of small $IB4^+$ neurons (n=157) and 64% of small $IB4^-$ neurons (n=226) expressed SNS/PN3 mRNA. In contrast to SNS/PN3, NaN was expressed predominantly in $IB4^+$ neurons ($p < 0.0001$, Mann-Whitney rank sum test) (Fig. 3b.), with 72% of small $IB4^+$ neurons (n=144) and only 38% of small $IB4^-$ neurons (n=98) expressing NaN mRNA. These results demonstrate that SNS/PN3 and NaN are differentially expressed in $IB4^+$ and $IB4^-$ DRG neurons; moreover, the distribution patterns indicate that SNS/PN3 and NaN must be coexpressed in a substantial subpopulation of $IB4^+$ DRG neurons, and possibly some $IB4^-$ neurons.

Example 2: *TTX-R currents in $IB4^+$ and $IB4^-$ neurons*

Since SNS/PN3 and NaN have different patterns of expression in small $IB4^+$ and $IB4^-$ neurons, whole-cell patch-clamp recordings from small $IB4^+$ and $IB4^-$ (17-32 μm diam.) DRG neurons were performed to determine if differences exist in voltage-dependence and kinetic properties of sodium currents in these cells. The neurons were maintained in culture for less than 30 hrs and binding to IB4 was determined prior to recording, permitting us to analyze approximately the same number of $IB4^+$ and $IB4^-$ neurons. Both fast inactivating ("fast", $\tau_h < 1$ msec at 0 mV) and slow inactivating ("slow", $\tau_h > 2.5$ msec at 0 mV) sodium currents were observed in small DRG neurons. The fast and slow sodium currents observed in the present study were similar to those previously described in small DRG neurons (Caffrey et al., 1992; Roy and Narahashi,

1992; Elliott and Elliott, 1993; Rizzo et al., 1994; Cummins and Waxman, 1997). All $IB4^+$ neurons (n=33) analyzed expressed slow sodium currents, and all but one of these $IB4^+$ cells also expressed fast sodium currents. In contrast, only 63% of the $IB4^-$ neurons (n=32) expressed slow currents, but all $IB4^-$ neurons expressed fast currents. Based on these observations, neurons were assigned to one of three groups: $IB4^+$ neurons with both fast and slow currents ($IB4^+$ F/S), $IB4^-$ neurons with both fast and slow currents ($IB4^-$ F/S), and $IB4^-$ neurons with only fast currents ($IB4^-$ F) (Fig. 4A).

Prepulse-inactivation was used (McLean et al., 1988; Roy and Narahashi, 1992; Elliott and Elliott, 1993; Cummins and Waxman, 1997) to separate fast and slow sodium current components in the cells. The peak amplitudes for fast and slow sodium current components are compared in Table 1. The slow current peak amplitude was similar for $IB4^+$ F/S and $IB4^-$ F/S cells, but the fast current amplitude was smaller for $IB4^+$ F/S cells than for the $IB4^-$ F/S cells. Because previous studies have shown that the fast current is TTX-sensitive (TTX-S) and the slow current is TTX-R, and because both SNS/PN3 and NaN putatively underlie TTX-R currents in DRG neurons, the properties of the slow currents in $IB4^+$ and $IB4^-$ cells were examined. The mean midpoints of activation and steady-state inactivation for slow currents were more negative for $IB4^+$ than for $IB4^-$ cells (Fig. 4C). While these differences are small, they are significant ($p < 0.005$). Figure 4B shows the distribution of inactivation midpoint values in individual $IB4^+$ and $IB4^-$ cells.

Table 1. Current amplitude: comparison of sodium currents in IB4$^+$ and IB4$^-$ neurons.

	IB4$^+$		IB4$^-$	
	with slow	w/o slow	with slow	w/o slow
0 nM TTX				
fast amplitude (nA)	29±2	---	46±10	31±4
slow amplitude (nA)	31±3	---	32±8	0.7±0.3
% cells	100%	0%	63%	37%
250 nM TTX				
TTX-R amplitude (nA)	38±4	---	37±7	1.0±0.2
% cells	100%	0%	60%	40%

Although the present inventors have previously shown that prepulse subtraction and TTX-subtraction give essentially the same results (Cummins and Waxman, 1997), indicating that all of the slow current is TTX-R and all of the fast current is TTX-S in DRG neurons, Scholz et al. (1998) observed a fast TTX-R current in young DRG neurons (7-21 days), raising the possibility that SNS/PN3 or NaN might encode a fast TTX-R current in DRG neurons. Therefore, sodium currents in $IB4^+$ and $IB4^-$ neurons in the presence of 250 nM TTX, which blocks 98% of the TTX-S current were also examined. Under these conditions, all $IB4^+$ cells (n=30) but only 60% of $IB4^-$ neurons (n=30) expressed large (>200 pA/pF) sodium currents. Thus, in agreement with the present inventors' prior classification, DRG neurons studied in the presence of 250 nM TTX can also be subdivided into three groups: $IB4^+$ with TTX-R sodium current, $IB4^-$ with TTX-R sodium current and $IB4^-$ without TTX-R sodium current (Fig. 5A, Table 1). The amplitude of the TTX-R current was similar for the $IB4^+$ and $IB4^-$ groups that expressed sodium currents.

The rate of inactivation (τ_h) for the TTX-R sodium currents in the $IB4^+$ and $IB4^-$ cells was compared. τ_h, measured at 0 mV, was significantly ($p < 0.05$) slower for the currents in $IB4^-$ cells (6.1 ±0.7 ms, n=18) than for the currents in $IB4^+$ cells (4.6 ±0.3 ms, n=30). However, while τ_h was longer in $IB4^-$ cells than in $IB4^+$ cells, all of the TTX-R currents in both groups had time constants greater than 2.5 ms and therefore are considered slow currents. Since all of the currents recorded in the presence of TTX were slow, and since fast TTX-R sodium currents were not observed in either $IB4^+$ cells or $IB4^-$ cells, the present inventors' data suggest that both NaN and SNS/PN3 encode slow TTX-R currents. It is interesting to note that the percentage of $IB4^-$ cells that expressed little or no sodium current in the presence of 250nM TTX was similar to the percentage of $IB4^-$ cells that expressed only fast sodium currents in the absence of TTX. Thus, as with previous studies (Kostyuk et al., 1981; McLean et al., 1988; Roy and Narahashi, 1992; Cummins and Waxman, 1997), the data are consistent with the fast currents being solely TTX-S and slow currents being solely TTX-R.

The midpoint of activation and steady-state inactivation of the TTX-R current was significantly ($p < 0.001$) more negative for $IB4^+$ neurons than for $IB4^-$ neurons that produce TTX-R sodium currents (Fig. 5B). The midpoint of steady-state inactivation ranged from

-31 to -44 mV for TTX-R currents in IB4$^+$ cells and from -25 to –36 mV for TTX-R currents in IB4$^-$ cells. Fig. 5C shows the distribution of the midpoints of activation and steady-state inactivation for the TTX-R currents. While 50% of IB4$^+$ cells have a midpoint of inactivation that is more negative than or equal to -37 mV, none of the IB4$^-$ cells do. Conversely, while 39% of the IB4$^-$ cells have a midpoint of inactivation that is positive to -31 mV, none of the IB4$^+$ cells do. However, while the extremes were dominated by IB4$^+$ cells and IB4$^-$ cells, respectively, there was overlap between the two groups.

Example 3: ***Effect of GDNF and NGF on SNS/PN3 mRNA expression***

The effect of GDNF and NGF on SNS/PN3 mRNA expression was examined in IB4$^+$ and IB4$^-$ neurons dissociated from adult L4/L5 DRG and cultured for 7 days with normal culture media ("control"), or with media supplemented with NGF, GDNF, BDNF or a combination of NGF and GDNF. DRG neurons dissociated and cultured in this manner, in the absence of exogenously added growth factors, have previously been shown to display changes in levels of sodium channel III and SNS/PN3 mRNAs similar to those seen 7 days after nerve transection *in vivo* (Dib-Hajj et al., 1996; Black et al., 1997). In agreement with a role for GDNF in the maintenance of IB4 reactivity in a subpopulation of small DRG neurons (Bennett et al., 1998b), the IB4 signal intensity was reduced, but discernable, in neurons maintained in cultures for 7 days without exogenously-added GDNF.

In comparison to 7 DIV control neurons, GDNF treatment significantly ($p_c < 0.01$) upregulated SNS/PN3 in both IB4$^+$ and IB4$^-$ neurons (Fig. 6). The effect of GDNF on SNS/PN3 mRNA expression was significantly ($p_c < 0.001$) more prominent in IB4$^+$ neurons (n=74) than in IB4$^-$ neurons (n= 51). Similar to GDNF treatment, NGF supplement significantly ($p_c < 0.001$) enhanced the SNS/PN3 hybridization signal in both IB4$^+$ (n=91) and IB4$^-$ (n=72) neurons compared to IB4$^+$ and IB4$^-$ 7 DIV control neurons (Fig. 6). However, unlike GDNF treatment, NGF did not preferentially upregulate (p = ns) SNS/PN3 in IB4$^-$ neurons (n=91) compared to IB4$^-$ neurons (n=72) (Fig. 6, 7). The combination of NGF and GDNF significantly increased the SNS/PN3 hybridization signal

in both $IB4^+$ and $IB4^-$ neurons compared to 7 DIV control neurons; however, the signal in $IB4^+$ neurons was less than that observed with either treatment alone, while $IB4^-$ neurons showed similar hybridization signals for the neurotrophins alone or in combination (Fig. 6). In contrast, it was observed that BDNF had no effect on SNS mRNA levels.

5

Example 4: ***NGF up-regulation of SNS/PN3 mRNA is blocked by K252a***

The up-regulation of SNS/PN3 by NGF could be mediated through the trkA pathway; alternatively, the upregulation may be through a pathway involving the p75 receptor. K252a, in the 100-400 nM range, is a potent inhibitor of NGF action through the 10 trk receptor and has been used to separate effects of NGF mediated through the high-affinity trkA receptor and the low affinity p75 receptor (Kase et al., 1987; Doherty and Walsh, 1989; Tapley et al., 1992; Kahle et al., 1994; Buck and Winter, 1996; De Bernardi et al., 1996). In control experiments without addition of K252a, exogenously-added NGF significantly ($p_c < 0.001$) increased the expression of SNS/PN3 mRNA in DRG neurons 15 maintained in culture for 7 DIV compared to neurons in culture for 7 DIV without added NGF (Fig.8). K252a blocked the effect of NGF on SNS/PN3 expression in a concentration-dependent manner at all concentrations of K252a tested (100- 400nM). The addition of K252a alone did not have a significant effect on SNS/PN3 mRNA expression compared to untreated controls (Fig. 8). These observations indicate that trkA is a 20 necessary component for the effect of NGF on the expression of SNS/PN3 mRNA. Since Schwann cells, which are present in the cultures, express p75 but not trkA (Yamamoto et al., 1993), these results argue that the effect of NGF on SNS/PN3 expression is mediated through a direct action involving TrkA on DRG neurons.

25 **Example 5:** ***Effect of GDNF and NGF on NaN mRNA***

While NaN expression is decreased following axotomy (Dib-Hajj et al., 1998b) the effect of culturing on NaN mRNA expression has not previously been established. As described for SNS, maintaining of DRG neurons in culture for 7 days without addition of growth factors (n=125) significantly ($p_c < 0.001$) reduced the levels of NaN mRNA 30 compared to freshly dissociated neurons (1 DIV) (n= 95). The reduction was, however,

limited to IB4$^+$ neurons, as no significant change was seen in IB4$^-$ neurons.

To examine if NaN mRNA is regulated by NGF and GDNF, NaN hybridization signals of DRG neurons treated in culture for 7 days with NGF (n=99), GDNF (n=122), BDNF (n= 93) or a combination of GDNF and NGF (n= 97) were compared to those in control cultures maintained for 7 days without addition of growth factors (n=125). In contrast to the effect on SNS/PN3 mRNA, NGF addition to the culture medium did not alter NaN levels in DRG neurons at 7 DIV in either IB4$^+$ (n= 45) or IB4$^-$ (n= 54) neurons (Fig. 9,10). In contrast, GDNF supplement to the culture medium significantly ($p_c <$ 0.001) increased NaN hybridization signal in IB4$^+$ neurons (n= 83) but had no effect on IB4$^-$ neurons (n= 39) (Fig. 9,10). These results indicate that GDNF, but not NGF, regulates the expression of NaN mRNA, and are consistent with a direct effect of GDNF on sensory neurons that express receptors for the GDNF–family of neurotrophins. When combining GDNF and NGF, the NaN hybridization was significantly ($p_c < 0.001$) increased in IB4$^+$ neurons (n= 57) compared to 7 DIV control neurons, whereas no significant change was seen in IB4$^-$ (n= 40) neurons. The hybridization signal in IB4$^+$ neurons treated with the combination of GDNF and NGF was somewhat lower than in cultures treated with GDNF alone (Fig. 9). BDNF had no significant effect on the levels of NaN expression compared to control

neurons.

Example 6: ***Effect of GDNF and NGF treatment on TTX-R currents***

Sodium currents in small DRG neurons after 7 DIV in the absence (control) or presence of exogenously added NGF, GDNF or combination NGF and GDNF was examined. For these experiments, IB4-reactivity after each cell was selected for recording was also determined. The staining obtained with FITC-labeled IB4 was much less intense at 7 DIV than that observed at 1 DIV. The intensity of IB4 fluorescence was clearly greater in the GDNF and GDNF/NGF groups than it was in the control and NGF groups at 7 DIV. In the absence of exogenously added GDNF, the reduced fluorescence intensity made it difficult to accurately classify the cells as IB4$^+$ or IB4$^-$. In contrast, IB4-reactivity in the *in situ* hybridization experiments (see above) was assessed with biotin-labeled IB4

and Cy2-conjugated streptavidin, which provide for enhanced signal amplification. Because of the uncertainty in the IB4-reactivity classification in the patch-clamp experiments on DRG neurons after 7 DIV, sodium current data was not subdivided into $IB4^+$ and $IB4^-$ cell groups .

Sodium currents were recorded in the presence of 250 nM TTX to isolate TTX-R currents. Figure 11A shows representative currents for each of the four groups of neurons. In the control group the amplitude of the TTX-R current was significantly lower (6.1±1.9 nA, n=41) than that recorded at 1 DIV (23.9±12.5 nA, n=36, Fig. 11B). This reduction in TTX-R currents following *in vitro* axotomy is very similar to that observed following *in vivo* axotomy (Cummins and Waxman, 1997). In neurons treated with NGF, the TTX-R current amplitude was similar (6.2±1.5 nA, n=40) to that observed in control neurons at 7 DIV. Neurons treated with GDNF, on the other hand, had significantly larger TTX-R currents (13.7±2.4 nA; n=40) than that of control neurons at 7 DIV ($p < 0.02$; Fig. 11B). The GDNF/NGF group had the largest peak current amplitude (16.1±2.4 nA, n= 40), although this was not significantly different from that of the GDNF group. As a measure of TTX-R current expression, the percentage of cells expressing TTX-R current amplitudes > 3 nA (Table II) was determined. In contrast to neurons at 1 DIV, where ~ 90 % of cells displayed TTX-R currents > 3 nA, at 7 DIV less than a quarter of the control cells had TTX-R current amplitudes > 3 nA. GDNF treatment was far superior to NGF treatment in increasing the proportion of cells with large TTX-R currents (Table II).

Because of the extensive neurites that develop in culture, a detailed characterization of the voltage-dependent and kinetic properties of the TTX-R currents in neurons at 7 DIV was not made. However, the TTX-R currents in all four groups appeared slow and the midpoints of activation and steady-state inactivation at 7 DIV were generally similar to those observed for $IB4^+$ cells at 1 DIV.

Table II:

	1 DIV	7 DIV			
		Control	NGF	GDNF	GDNF/NGF
% cells with I_{Na} > 3 nA:	91±5%	24±7%	38±8%	60±8%	68±7%
cell capacitance:	27±2pF	30±2pF	28±2pF	33±2pF	35±2pF
number cells:	36	41	40	40	40

Example 7: ***Effect of GDNF and NGF on TTX-R currents in small DRG neurons from SNS-null mice***

Small DRG neurons were taken from SNS-null mice (Akopian et al., 1999) and cultured *in vitro* using the methods described for rat neurons above. Neurons were cultured in the presence of 250 nM TTX and further in the presence or absence of GDNF (10ng/ml) or NGF (50 ng/ml). The currents were elicited by 200 ms test pulses to potentials ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. As shown in Figures 12A, in untreated cultures the persistent non-SNS TTX-R sodium current traces were drastically decreased after 7 days in vitro (7 DIV). By contrast, in GDNF-treated cultures the TTX-R currents were maintained at control levels. Similarly, persistent non-SNS TTX-R peak currents were returned to control levels by the addition of GDNF, as shown in Figure 12B. Notably, the addition of NGF to the culture medium did not significantly effect the size of persistent non-TTX-R current compared to the control 7 DIV neurons.

Example 8: ***GDNF attenuates the decrease of TTX-R current density and persistent Na^+ currents*** **in vivo**

Uninjured neurons predominantly express slow-inactivating TTX-R and slowly-repriming TTX-S Na^+ currents. Following axotomy, TTX-R current density is greatly reduced and rapidly repriming TTX-S currents predominate. Consequently, the effect *in vivo* of exogenously administered GDNF delivered to transected nerves on sodium currents in small DRG neurons was examined. GDNF (1.2 μg/day per animal) was delivered to DRG neurons *in vivo* via an osmotic pump attached to the transected sciatic nerve. (Dib-Hajj et al. 1998a). The opposite sciatic nerve was also transected and hooked to a pump containing only Ringer's solution.

As shown in Figures 13A and 13B, both slowly-inactivating and persistent TTX-R currents were partially restored to toward control levels in GDNF-treated axotomized

neurons. It was also observed that TTX-S currents in GDNF-treated axotomized neurons expressed intermediate repriming kinetics. Thus, GDNF treatment can mitigate the effects of axotomy on the sodium currents of a sub-population of small DRG sensory neurons.

Although the present invention has been described in detail with reference to the examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

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CLAIMS

1. A method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF that is effective to alter TTX-R Na^+ current flow through NaN sodium channels in DRG or trigeminal neurons.

2. The method of claim 1, wherein the sensory neuron is a DRG or trigeminal neuron.

3. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in sensory neurons.

4. The method of claim 3, wherein the sensory neuron is a DRG or trigeminal neuron.

5. The method of claim 3 or 4, wherein the TTX-R sodium channels are selected from the group consisting of SNS/PN3 and NaN channels.

6. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and NaN channels.

7. The method of claim 6, wherein the agent is a neurotrophin.

8. The method of claim 6, wherein the neurotrophin is selected from the group

consisting of NGF and GDNF and or other members of their families.

9. The method of claim 6, wherein the agent modulates the production or activity of a neurotrophin that modulates the activity of the sodium channel.

10. The method of claim 9, wherein the agent modulates the level or activity of GDNF or NGF.

11. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

12. A method of identifying an agent which modulates TTX-R Na^+ current through NaN channels comprising the step of:

determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

13. The method of claim 10, wherein the agent modulates the GDNF induction of NaN.

14. A cell that has been transformed to express a functional recombinant GDNF receptor and, optionally, recombinant NaN.

15. A method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na^+ channels.

16. The method of claim 15, wherein the cell is the transformed cell of claim 12.

17. The method of claim 15, wherein the Na^+ channel is selected from the group consisting of the SNS/PN3 and NaN channels.

18. The method of claim 15, wherein the cell does not express SNS.

19. The method of claim 15, wherein the cell is present in a living animal.

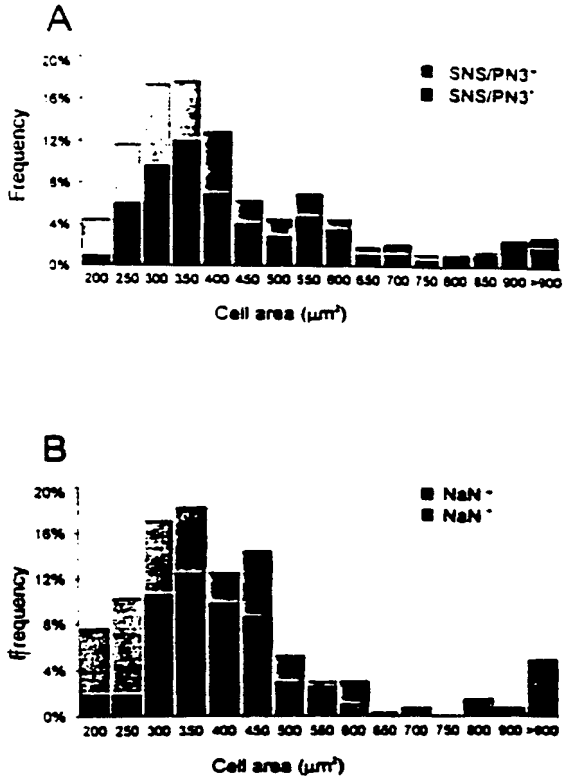

A
SNS/PN3-
SNS/PN3-
Frequency
20%
16%
12%
8%
4%
0%
200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 >900
Cell area (μm²)
B
NaN-
NaN-
frequency
20%
16%
12%
8%
4%
0%
200 250 300 350 400 450 500 550 600 650 700 750 800 900 >900
Cell area (μm²)

FIG. 1

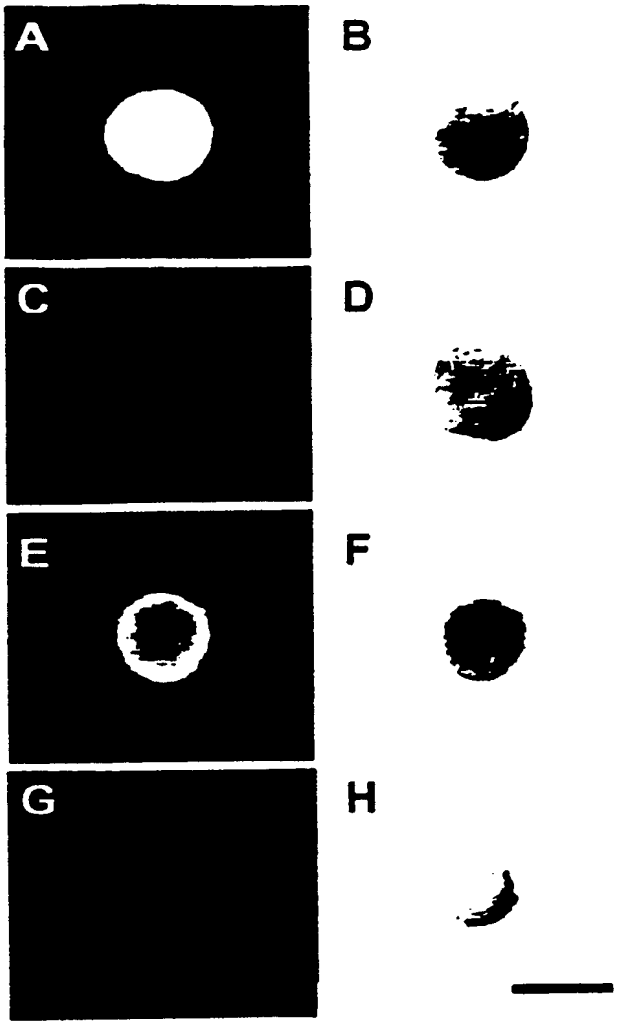


FIG. 2

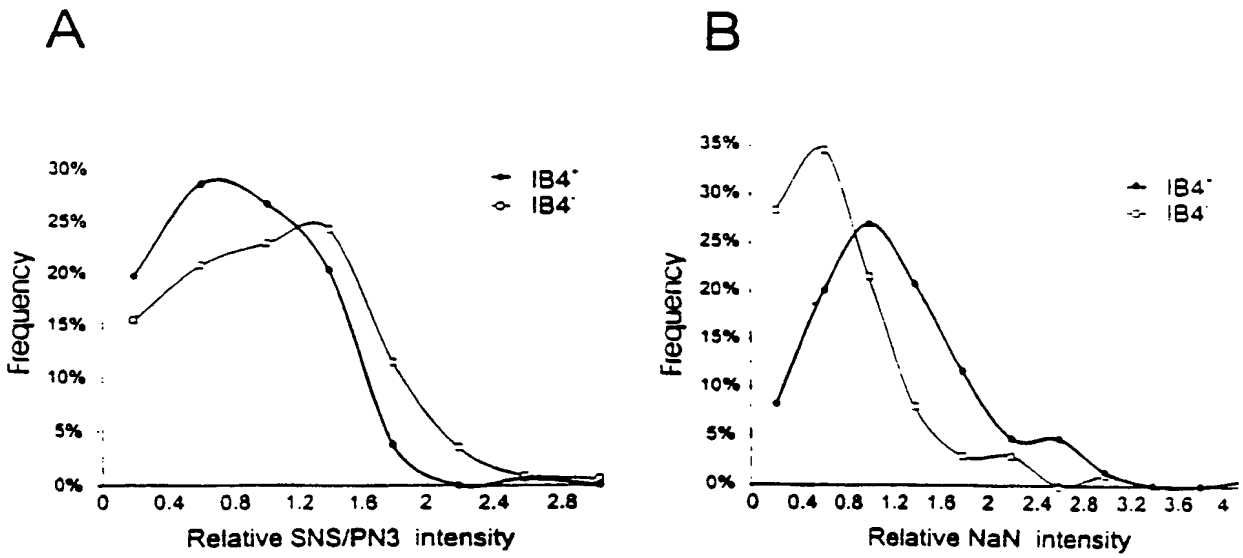

A
B
30%
25%
20%
15%
10%
5%
0%
Frequency
IB4+
IB4-
0 0.4 0.8 1.2 1.6 2 2.4 2.8
Relative SNS/PN3 intensity
35%
30%
25%
20%
15%
10%
5%
0%
Frequency
IB4+
IB4-
0 0.4 0.8 1.2 1.6 2 2.4 2.8 3.2 3.6 4
Relative NaN intensity

FIG. 3

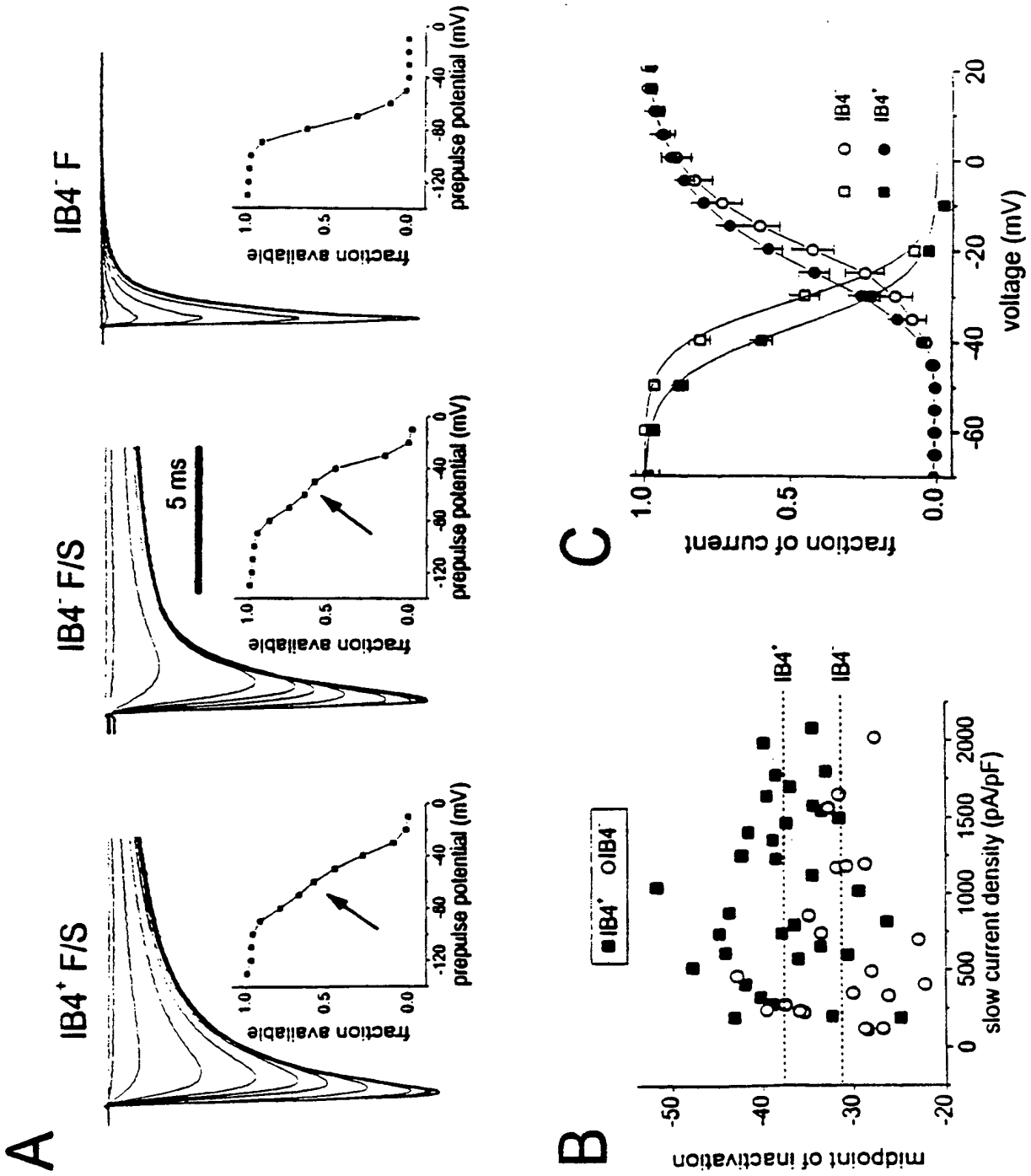

A
IB4+ F/S
IB4- F/S
IB4- F
5 ms
fraction available
prepulse potential (mV)
B
IB4+ oIB4-
IB4+
IB4-
midpoint of inactivation
slow current density (pA/pF)
C
IB4-
IB4+
fraction of current
voltage (mV)

FIG. 4

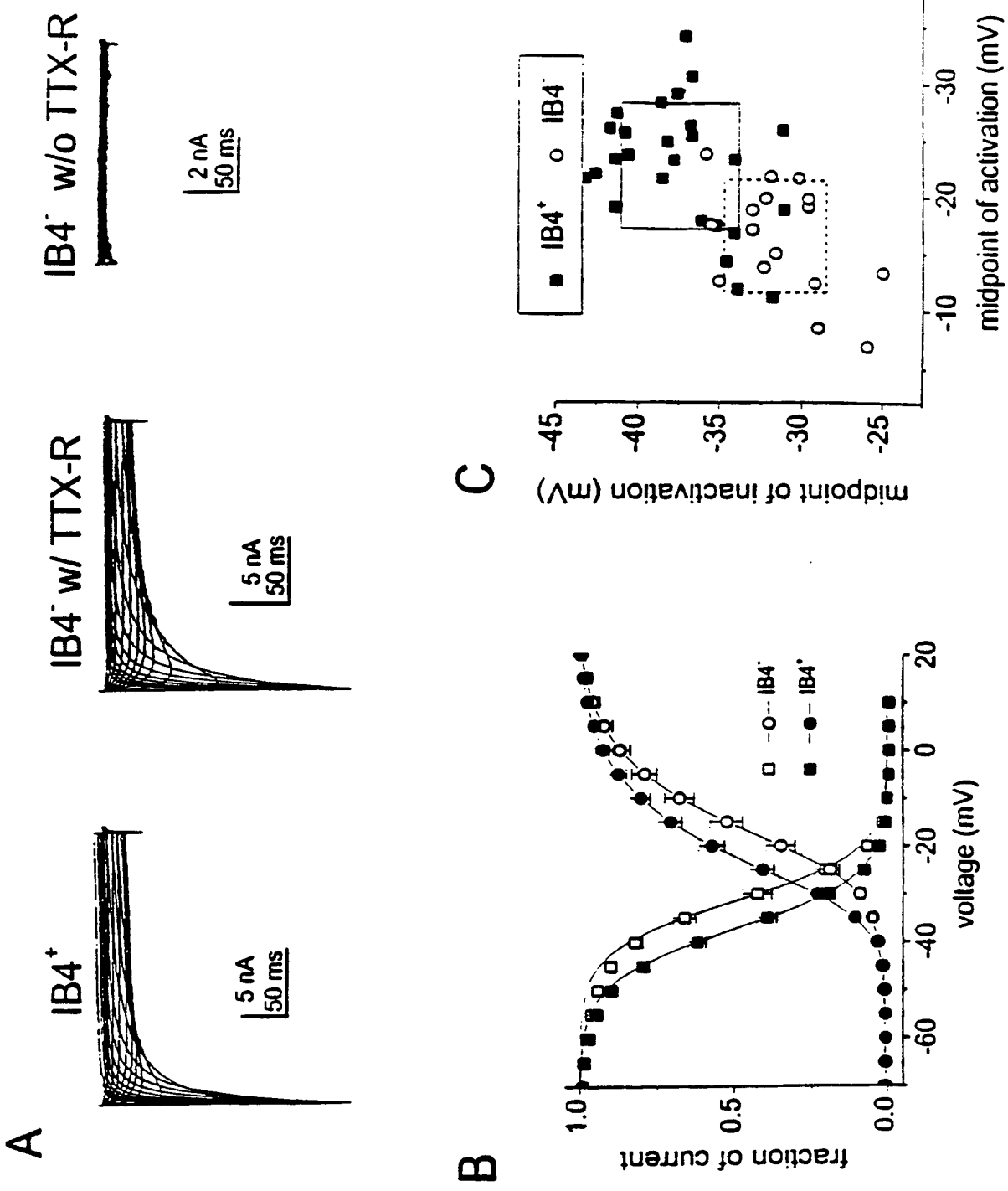

A
IB4+
5 nA
50 ms
IB4- w/ TTX-R
5 nA
50 ms
IB4- w/o TTX-R
2 nA
50 ms
B
fraction of current
1.0
0.5
0.0
-60
-40
-20
0
20
voltage (mV)
IB4-
IB4+
C
midpoint of inactivation (mV)
-45
-40
-35
-30
-25
IB4+
IB4-
midpoint of activation (mV)
-40
-30
-20
-10

FIG. 5

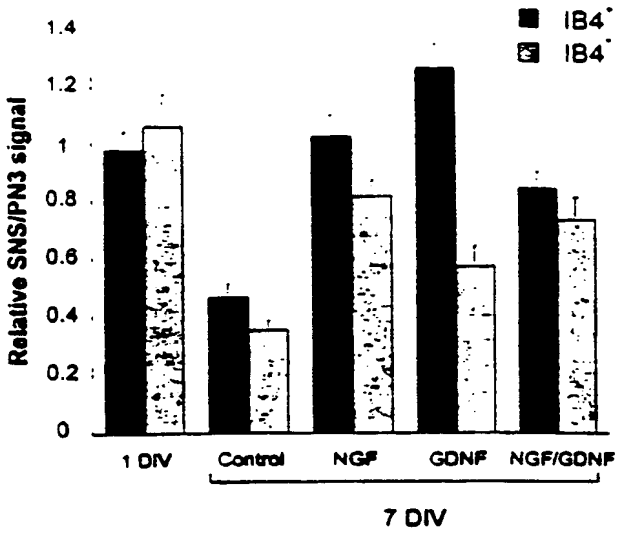

IB4+
IB4-
Relative SNS/PN3 signal
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 DIV
Control
NGF
GDNF
NGF/GDNF
7 DIV

FIG. 6

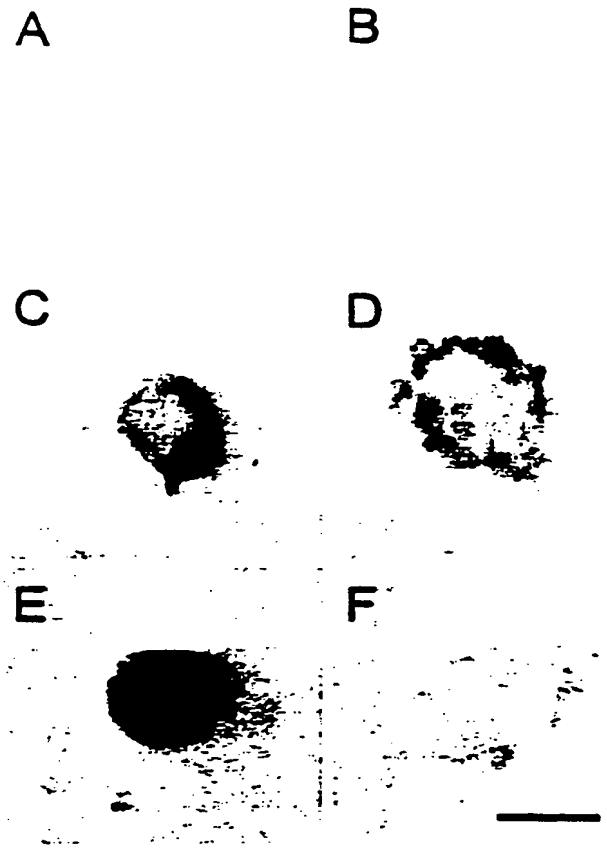


FIG. 7

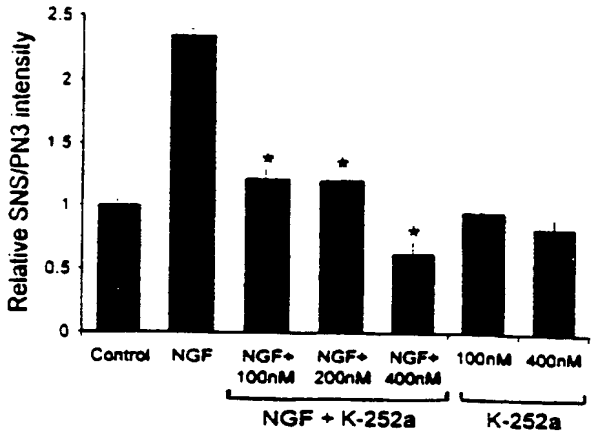

Relative SNS/PN3 intensity
2.5
2
1.5
1
0.5
0
Control
NGF
NGF+ 100nM
NGF+ 200nM
NGF+ 400nM
100nM
400nM
NGF + K-252a
K-252a

FIG. 8

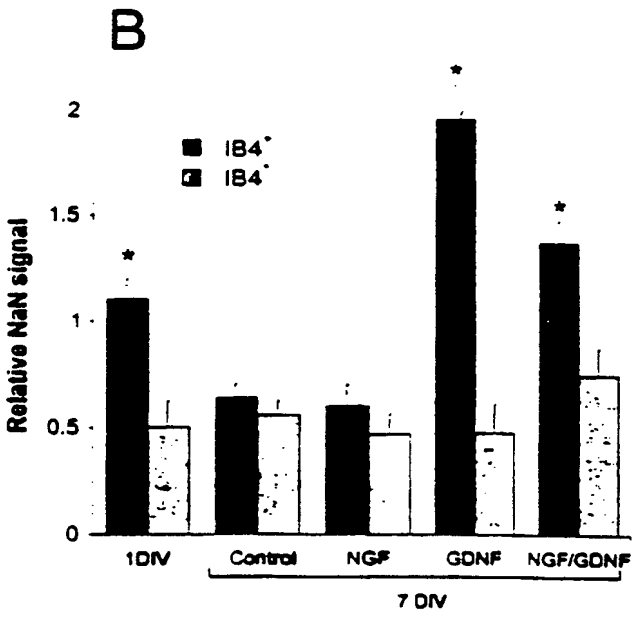

B
IB4+
IB4-
Relative NaN signal
2
1.5
1
0.5
0
1DIV
Control
NGF
GDNF
NGF/GDNF
7 DIV

FIG. 9

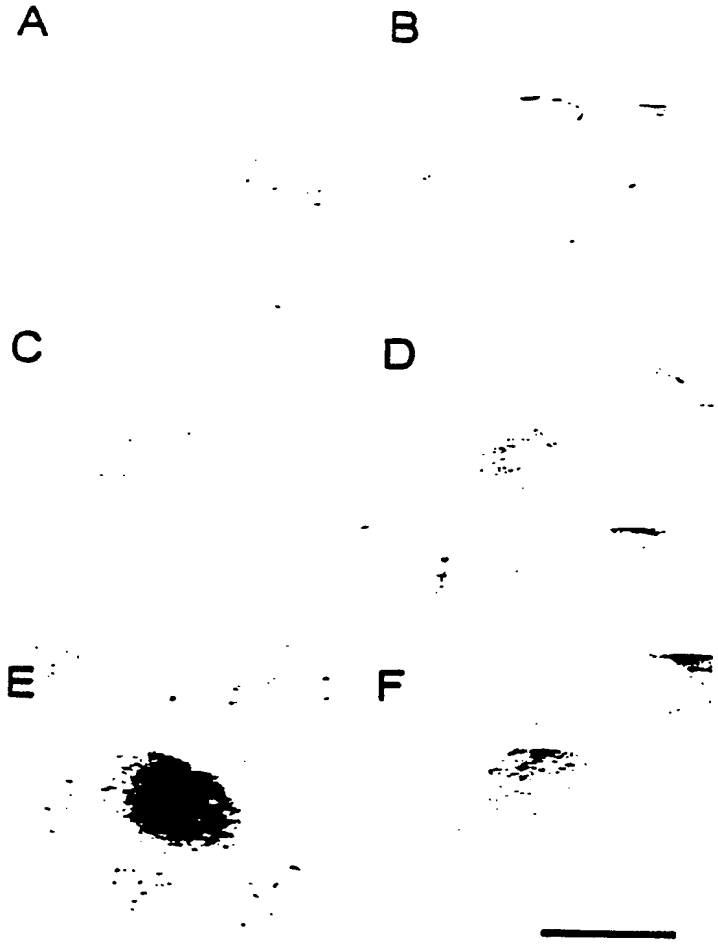


FIG. 10

FIG. 11

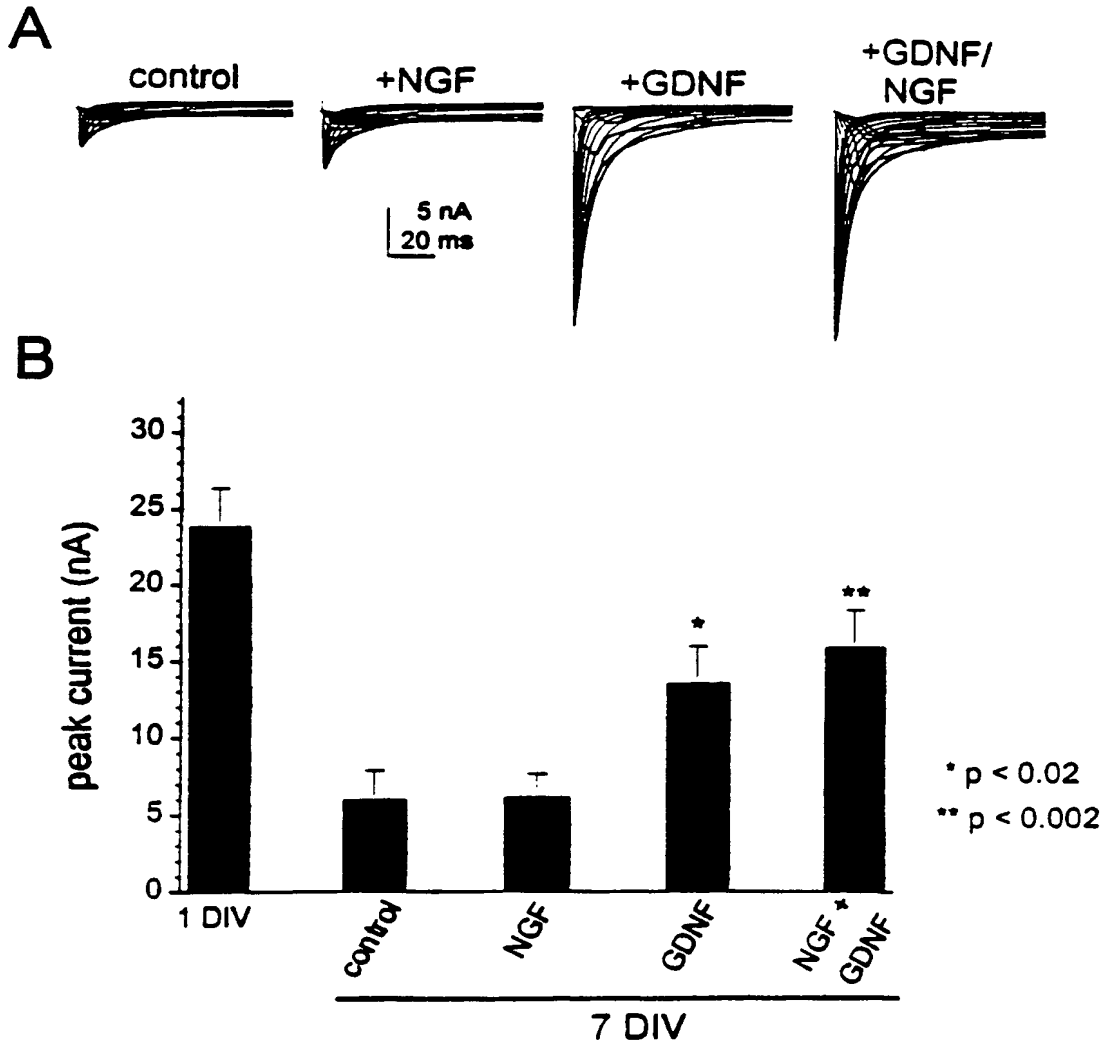

A
control
+NGF
+GDNF
+GDNF/
NGF
5 nA
20 ms
B
peak current (nA)
30
25
20
15
10
5
0
1 DIV
control
NGF
GDNF
NGF + GDNF
7 DIV
* p < 0.02
** p < 0.002

Figure 12A

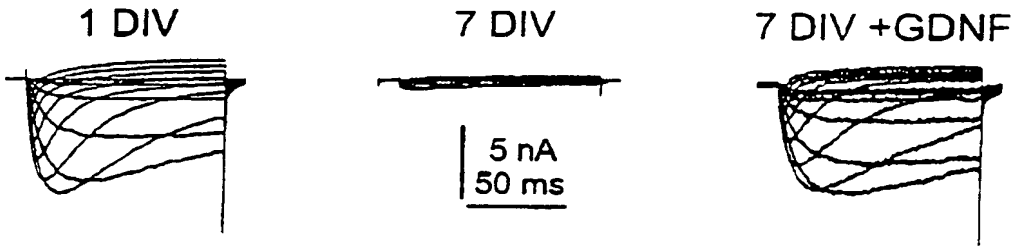
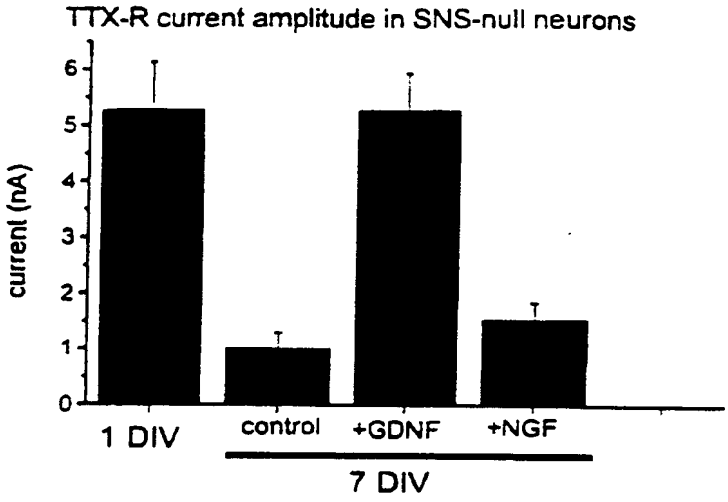


Figure 12B



a. Axotomy down-regulates TTX-R current density.
b. GDNF-treatment attenuates this decrease.

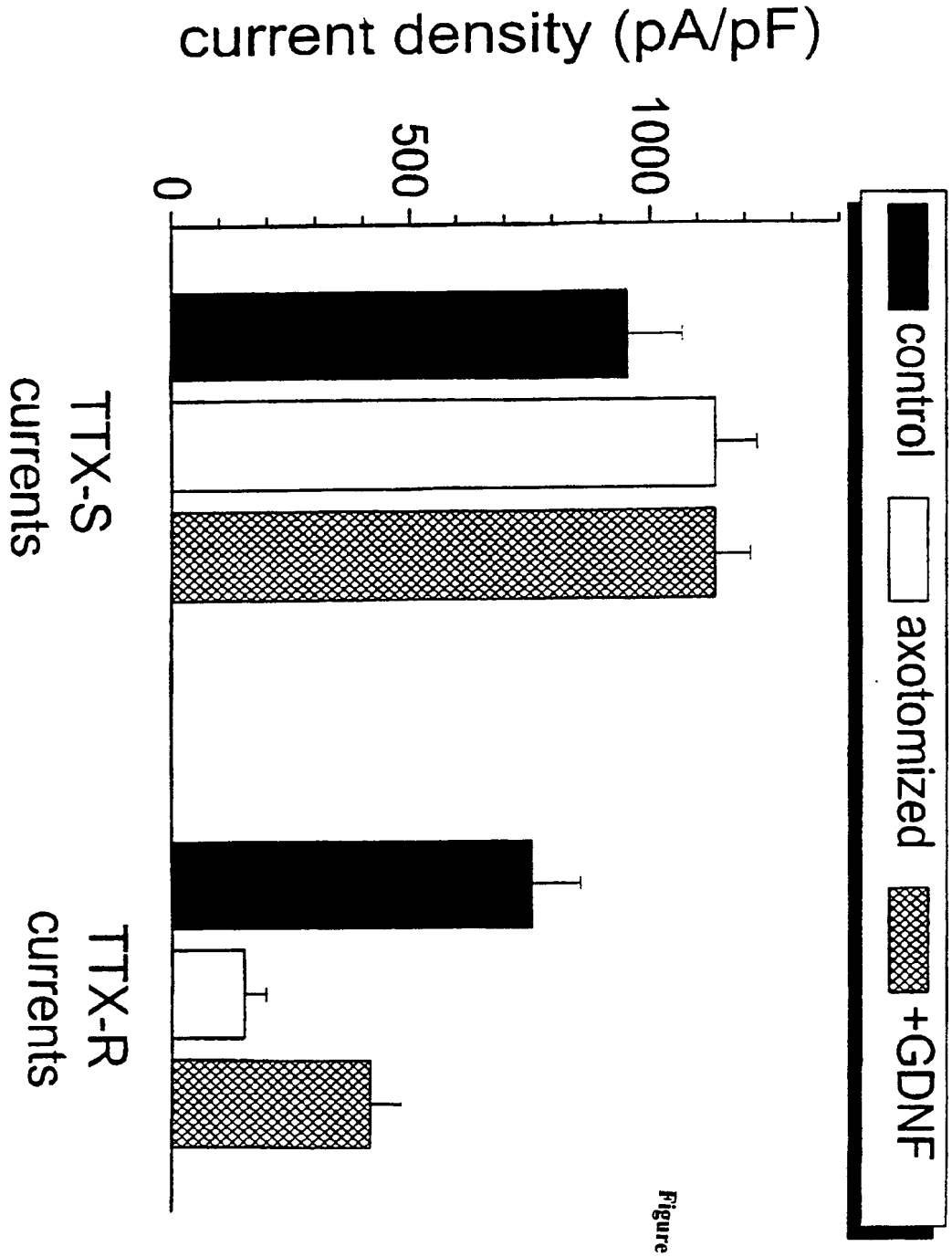


Figure 13A

a. Axotomy decreases persistent Na^+ currents
b. GDNF-treatment attenuates this decrease

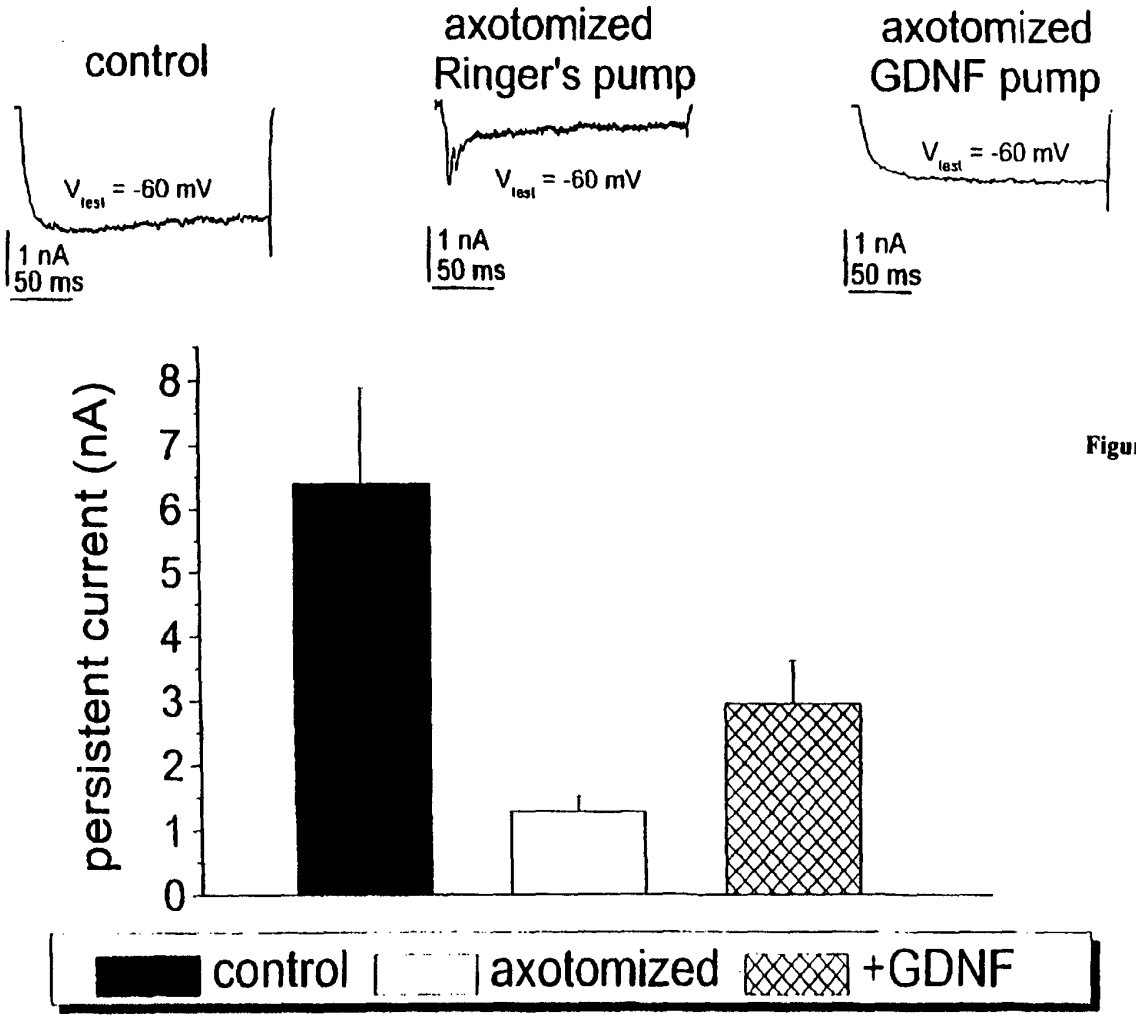


Figure 13B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/00, 38/16, 38/17, 38/18, 38/22
US CL :514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,731,284 A (WILLIAMS) 24 March 1998(24.03.98), columns 16-19.	1-2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 MARCH 2000	Date of mailing of the international search report 18APR2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL PAK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BRS, STN, MEDLINE

search terms: GDNF, growth factor, sodium channel, dorsal root ganglia

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-2, drawn to a method to treat pain by administering GDNF to alter Na current flow.
Group II, claim(s) 3-5, drawn to a method to treat pain by administering GDNF to restore Na channels.
Group III, claim(s) 6-8, drawn to a method to treat pain by administering an agent to modulate transcription and translation of mRNA.
Group IV, claim(s) 9-13, drawn to a method to treat pain by administering an agent to modulate GDNF.
Group V, claim(s) 14, drawn to a cell.
Group VI, claim(s) 15-19, drawn to a method to screen candidate compound.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features because the method of claim 1 is anticipated by WILLIAMS (US 5,731,284 A (WILLIAMS) 24 March 1998) and thus, does not share a special technical feature with any other group.

The methods of Groups II-IV and VI, do not share the same or corresponding special technical feature with Group I, because the methods have materially different process steps and are practiced for materially different purposes, and each defines a separate invention over the art.

The product of Group V does not share the same or corresponding special technical feature with Group I, because the product of Group V can be used in a materially different process of protein purification.

Since Groups I-VI do not share a special technical feature, unity of invention is lacking.

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(54) Title: EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

(57) Abstract: The present invention provides a new means for altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes methods to treat pain or hyperexcitability phenomena by altering or modulating the sodium channel expression or activity. The present invention also includes methods of identifying agents which modulate TTX-R Na^+ current through channels, particularly NaN channels, by altering or modulating the level or activity of the channel or by altering or modulating the expression or activity of a neurotrophin.

EFFECTS OF GDNF AND NGF ON SODIUM CHANNELS IN DRG NEURONS

RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Application Serial No. 09/354,147, filed July 16, 1999, which is a continuation-in-part of PCT International Application No. PCT/US99/02008, also entitled "Modulation of Sodium Channels of Dorsal Root Ganglia," filed January 29, 1999, each of which are related to U.S. Provisional Application 60/072,990, filed January 29, 1998, U.S. Provisional Application 60/109,402 entitled "Modulation of Sodium Channels in Dorsal Root Ganglia", filed November 20, 1998 and to U.S. Provision Application 60/109,666, entitled "Differential Role of GDNF and NGF in the Maintenance of Two TTX-Resistant Sodium Channels in Adult DRG Neurons," filed on November 20, 1998, all of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to the role of glial-derived nerve factor (GDNF) and Nerve Growth Factor (NGF) in modulating the activity of tetrodotoxin (TTX)-resistant sodium channels in dorsal root ganglion (DRG) neurons.

BACKGROUND

Small dorsal root ganglion (DRG) neurons give rise to c- and Aδ-fibers and are predominantly nociceptive (Lynn and Carpenter, 1982; Kress et al., 1992). Many of these neurons display somatic sodium current components that are relatively resistant to tetrodotoxin (TTX) (Kostyuk et al., 1982; Roy and Narahashi, 1992), and it has been suggested that TTX-resistant (TTX-R) sodium currents play an important role in nociceptive transmission (Jeftinija, 1994; Gold et al., 1996). Transcripts for two TTX-R sodium channels, SNS/PN3 and NaN, are preferentially expressed in small DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al., 1998b), and may be

responsible for the TTX-R sodium current observed in the soma (Kostyuk et al., 1981; Bossou and Feltz, 1984; McLean et al., 1988; Caffrey et al., 1992; Roy and Narahashi, 1992) and c-fibers (Quasthoff et al., 1995) of these neurons.

Following transection of the sciatic nerve, TTX-R sodium currents in DRG neurons are attenuated (Rizzo et al., 1995; Cummins and Waxman, 1997) and concomitantly SNS/PN3 (Dib-Hajj et al., 1996; Okuse et al., 1997) and NaN (Dib-Hajj et al., 1998b) transcripts are down-regulated. Axotomy prevents the retrograde transport of nerve growth factor (NGF) from peripheral targets and this may account for many of the phenotypic changes that appear in DRG neurons following axotomy (for review see Verge et al., 1996). In agreement with a role for NGF in maintaining TTX-R sodium currents, infusion of NGF to the transected nerve stump restores SNS/PN3 mRNA to near-normal levels, but only partially rescues TTX-R currents (Dib-Hajj et al., 1998a).

Several mechanisms may explain why NGF only partially restores TTX-R currents following axotomy; an intriguing possibility, however, is that neurotrophins other than NGF regulate the expression of TTX-R sodium channels in the subpopulation of DRG neurons that lack receptors for NGF. Glial cell line-derived neurotrophic factor (GDNF) has been suggested to be important for the maintenance of phenotypic properties in the subset of small sensory neurons that lack NGF receptors (Molliver et al., 1997; Bennett et al., 1998b), and intrathecal administration of GDNF can ameliorate the reduction in conduction velocity in small-diameter axons after sciatic nerve transection (Bennett et al., 1998b). Although a substantial body of evidence demonstrates a role for NGF in the regulation of specific sodium (Kalman et al., 1990; D'Arcangelo et al., 1993; Zur et al., 1995) and potassium (Sharma et al., 1993; Lesser and Lo, 1995) channel expression, the actions of GDNF on ion channel expression has not been established.

GDNF-sensitive and NGF-sensitive neurons can be differentiated by their different ability to bind the lectin IB4 from *Griffonia simplicifolia*. IB4-binding ($IB4^+$) neurons express the receptor/transducing elements necessary to respond to GDNF whereas $IB4^-$ neurons generally express the NGF receptors, TrkA and p75 (Averill et al., 1995; Wright and Snider, 1995; Bennett et al., 1996; Molliver et al., 1997; Bennett et al., 1998b). Based on differences in terminal fields in the spinal cord and certain differences in phenotype of $IB4^+$ and $IB4^-$ neurons, it has been suggested that these two subpopulations of small DRG

neurons may play distinct roles in nociceptive transmission (see Snider and McMahon, 1998).

In the present study, the expression of SNS/PN3 and NaN in $IB4^+$ and $IB4^-$ DRG neurons was examined to determine 1) whether subpopulations of small DRG neurons express distinct TTX-R sodium channels, with different current characteristics and 2) whether NGF and GDNF have differential effects on the expression of SNS/PN3 and NaN, and on TTX-R currents. Inappropriate electrical activity may be involved in some pain syndromes (Matzner and Devor, 19940, and the expression and differential regulation of specific sodium channel gene products in selected sensory neurons may have important implications for pharmaceutical management of pain. Consequently, there is a need for compositions that modulate expression of these genes and also for methods to identify and use the such compositions.

SUMMARY OF THE INVENTION

The present invention provides a new means of altering or modulating inappropriate electrical activity which may be involved in pain syndromes. The present invention includes a method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF or GDNF-related molecule that is effective to alter TTX-R Na^+ current flow through NaN sodium channels in sensory neurons such as DRG or trigeminal neurons.

The present invention also includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF or a GDNF-related molecule that is capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in sensory neurons such as DRG or trigeminal neurons.

In another embodiment, the invention includes a method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and NaN channels. Such agents includes neurotrophins such as NGF and GDNF.

In another embodiment, the invention includes a method to treat pain, paraesthesia

or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

Another aspect of the invention includes a method of identifying an agent which modulates TTX-R Na^+ current through NaN channels comprising the step of determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

Also, the invention includes a method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na^+ channels.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

Figure 1. Cell size distribution of DRG neurons positive and negative for SNS/PN3 (A) and NaN mRNA (B). Data for each graph were pooled from four independent experiments by dividing the OD of each neuron by the mean OD of all neurons captured in that experiment. Neurons with a relative intensity > 0.8 are considered positive for SNS/PN3 or NaN. For each size bin, the graphs present the percent of all neurons that are positive and negative for SNS/PN3 (A) or NaN (B). n = 413 for SNS/PN3. n = 263 for NaN. Both SNS/PN3 and NaN are expressed in a large proportion of small DRG neurons.

Figure 2. SNS/PN3 and NaN mRNA in representative $IB4^+$ and $IB4^-$ DRG neurons. IB4 binding, visualized using biotin-labeled IB4 and Cy2-labeled streptavidin, can be readily recognized after *in situ* hybridization. Examples of 1 day *in vitro* ("DIV") neurons processed for *in situ* hybridization and IB4-binding are shown. A,C) IB4 positivity and negativity after SNS/PN3 *in situ* hybridization. B,D) Corresponding Nomarski images of the same neurons. SNS/PN3 mRNA is expressed in both $IB4^+$ (A,B) and $IB4^-$ (C,D) DRG neurons. E,G) IB4 binding after NaN *in situ* hybridization. F,H) Corresponding Nomarski images of the same neurons. NaN mRNA is expressed predominantly in $IB4^+$ neurons (E, F), whereas NaN was undetectable in many $IB4^-$ neurons (G,H). Scale bar is 25 μm.

Figure 3. SNS/PN3 (A) and NaN (B) distribution in small (<30 μm) $IB4^+$ and $IB4^-$

neurons. (A,B) Frequency diagram showing the percentage of $IB4^+$ and $IB4^-$ neurons, respectively, within each hybridization intensity bin (bin-width 0.4 unit). The percentage of neurons is plotted at the midpoint of each bin. The relative intensity was calculated by subtracting the background intensity and then dividing the optical density of each neurons by the mean of all neurons examined in that experiment. Data was pooled from four independent experiments. *A)* SNS/PN3 mRNA is expressed in both in $IB4^+$ and $IB4^-$ DRG neurons. Note that the most intensely stained cells (relative intensity >2.0) were almost exclusively $IB4^-$. The difference in SNS/PN3 hybridization signal between $IB4^+$ and $IB4^-$ neurons was significant ($p< 0.05$, n = 338, Mann-Whitney Rank sum test). *B)* NaN is expressed predominantly in $IB4^+$ DRG neurons. The difference in NaN hybridization signal between $IB4^+$ and $IB4^-$ neurons was significant ($p< 0.001$, n = 242, Mann-Whitney Rank sum test).

Figure 4. Comparison of sodium currents in $IB4^+$ and $IB4^-$ DRG neurons. A) Families of current traces recorded from representative neurons without TTX in the bath are shown. While 97% of the $IB4^+$ neurons exhibited both fast and slow currents (left panel), only 63% of $IB4^-$ neurons exhibited both fast and slow currents (middle panel). 37% of $IB4^-$ neurons exhibited predominantly fast currents (left panel). The currents were elicited by 20 ms test pulses to -10 mV after 500 ms prepulses to potentials over the range of -130 mV to -10 mV. The inset graph in each panel shows the corresponding steady-state inactivation curves for each cell. Current is plotted as a fraction of peak current. Two current components can be easily resolved in the left and middle panels; a slowly inactivating component that has a relatively depolarized voltage-dependence of inactivation (V_h) and a fast inactivating component that has a more negative V_h. The steady-state inactivation curves for these cells are bimodal because of the different inactivation properties of the two components (arrows indicate point of inflection). The $IB4^-$ cell in the right panel, on the other hand, appears to exhibit only fast-inactivating currents and the steady-state inactivation is not inflected. B) The midpoints of steady-state inactivation for the slow current component in $IB4^+$ (solid squares, n=33) and $IB4^-$ F/S (open circles, n=20) neurons are plotted as a function of the slow current density. The fast currents were eliminated using prepulse inactivation. The horizontal dashed lines indicate the average midpoint of inactivation for the slow currents in $IB4^+$ neurons (-37.7±1.0 mV)

and IB4$^-$ F/S neurons (-31.3±1.1 mV). C) Normalized activation (circles) and steady-state inactivation (squares) curves for the slow currents in IB4$^+$ (filled symbols, n=33), and IB4$^-$ (open symbols, n=20) neurons are shown. Cells were held at -100 mV, and prepulsed to -50 mV to inactivate TTX-S currents. Activation was measured with 40 ms test pulses ranging from -70 to +40 mV in 5 mV steps and the midpoint of activation was -23.6±1.3 mV for IB4$^+$ neurons and -17.4±1.6 mV for IB4$^-$ neurons. Steady-state inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. Error bars indicate standard error.

Figure 5. Comparison of TTX-R currents in IB4$^+$ and IB4$^-$ neurons. A) Families of voltage-activated TTX-R current traces recorded from representative IB4$^+$ and IB4$^-$ neurons with 250 nM TTX in the bath are shown. While all of the IB4$^+$ neurons exhibited large (>3 nA) TTX-R currents (left panel; n=30), 60% of the IB4$^-$ exhibited large TTX-R currents (middle panel; n=18) and 40% of the IB4$^-$ cells exhibited little or no TTX-R current (right panel; n=12). The currents were elicited by 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. B) Normalized activation (circles) and steady-state inactivation (squares) curves for the TTX-R currents in IB4$^+$ (filled symbols, n=30), and IB4$^-$ (open symbols, n=18) neurons are shown. Error bars indicate standard error. Cells were held at -100 mV and activation was measured with 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Steady-state fast inactivation was measured with 500 ms prepulses and 20 ms test pulses to -10 mV. C) Scatterplots showing the midpoint of steady-state inactivation against the midpoint of activation for TTX-R currents in IB4$^+$ (filled squares) and IB4$^-$ (open circles) neurons. The boxes delineate the mean ± standard deviation for the IB4$^+$ (solid outline) and IB4$^-$ (dashed outline) data.

Figure 6. Effects of neurotrophins on the expression of SNS/PN3 mRNA. The optical densities of neurons captured from three separate experiments were normalized and pooled. The graph represents the mean normalized OD for IB4$^+$ and IB4$^-$ neurons respectively for each condition. Error bar represents standard error. All treatments produced a significant difference in the respective subpopulations ($p_c < 0.01$; Bonferroni *t* test for multiple comparisons), compared to 7 DIV control neurons. Whereas GDNF was significantly ($p_c <$

0.001) more effective on IB4$^+$ neurons compared to IB4$^-$ neurons, no significant difference (p_c = ns) was observed between IB4$^+$ and IB4$^-$ neurons after NGF treatment.

Figure 7. SNS/PN3 *in situ* hybridization of representative IB4$^+$ and IB4$^-$ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4$^+$ A) or IB4$^-$ (B) neurons. NGF increases the signal in both IB4$^+$ (C) and IB4$^-$ (D) neurons. GDNF increased the hybridization signal predominantly in IB4$^+$ neurons (E), with little effect on IB4$^-$ neurons (F). Scale bar is 25 μm.

Figure 8. The upregulation of SNS/PN3 mRNA by NGF is blocked by K252a. The diagram shows the relative SNS/PN3 signal in neurons treated with NGF alone (50 ng/ml; n= 309), or in combination with K252a at 100nM (n= 151), 200nM (n=248) or 400nM (n=65). Each bar represents the mean of the pooled, normalized optical densities from 2 to 4 independent experiments. Error bars represent standard error. K252a alone does not affect the SNS/PN3 hybridization signal intensity at these concentrations (Control: n= 216; 100nM K252a: n= 156; 400nM K252a: n= 86). *The SNS/PN3 hybridization signal was significantly (p_c< 0.001; Bonferroni *t* test) less intense in neurons treated with both NGF and K252a compared to neurons treated with NGF alone.

Figure 9. Effects of neurotrophins on the expression of NaN mRNA. Data from three different experiments are normalized and pooled. The graph represents the mean normalized OD for IB4$^+$ and IB4$^-$ neurons respectively for each condition. Error bar represents standard error. At 7 DIV the NaN hybridization signal was significantly reduced in IB4$^+$ neurons. GDNF increased the NaN hybridization signal in IB4$^+$ neurons, but had no effect on in IB4$^-$ neurons. NGF did not increase NaN expression in either IB4$^+$ or IB4$^-$ neurons. *Significantly different from 7 DIV control by Bonferroni *t* test for multiple comparisons (p_c < 0.001).

Figure 10. NaN *in situ* hybridization of IB4$^+$ and IB4$^-$ DRG neurons after treatment with NGF and GDNF for 7 days. Without addition of either growth factor, little hybridization signal is seen in either IB4$^+$ A) or IB4$^-$ (B) neurons. NGF did not increase the signal in either IB4$^+$ (C) or IB4$^-$ (D) neurons. GDNF increased the hybridization signal markedly in IB4$^+$ neurons (E), but had no effect on IB4$^-$ neurons (F). Scale bar is 25 μm.

Figure 11. GDNF increases TTX-R currents in cultured DRG neurons. A)

Families of voltage-activated TTX-R current traces recorded from representative DRG neurons. Currents from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons are shown. Cells were studied after 7 DIV. The bath solution contained 250 nM TTX. The currents were elicited by 200 ms test pulses ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. B) TTX-R peak current amplitude from control, NGF-treated, GDNF-treated and GDNF+NGF-treated neurons after 7DIV are shown. For comparison, the TTX-R peak current amplitude measured at 1 DIV is also shown. GDNF treatment significantly increases the size of the TTX-R current compared to the control 7 DIV neurons.

Figure 12. Figure 12A shows families of voltage-activated TTX-R current traces recorded from representative SNS-null neurons after 1 day in vitro (1DIV), untreated SNS-null neurons after 7 days in vitro (1DIV) and GDNF-treated SNS-null neurons after 7 days in vitro (DIV). Figure 12B shows TTX-R peak current amplitude from 1DIV, untreated 7DIV, GDNF-treated 7DIV and NGF-treated 7DIV SNS-null neurons.

Figure 13. Figure 13A shows that GDNF administration attenuates the decrease in current density that results from axotomy. Figure 13B shows that GDNF administration attenuates the decrease in persistent Na^+ currents that results from axotomy.

DETAILED DESCRIPTION

Following axotomy, electrophysiological properties of small dorsal root ganglion (DRG) neurons are markedly altered, with attenuation of TTX-R sodium currents and the appearance of rapidly repriming TTX-S currents. The reduction in TTX-R currents has been attributed to a down-regulation of sodium channels SNS/PN3 and NaN. While infusion of exogenous NGF to the transected nerve restores SNS/PN3 transcripts to near-normal levels in small DRG neurons, TTX-R sodium currents are only partially rescued. Binding of the isolectin IB4 distinguishes between two major subpopulations of small DRG neurons: $IB4^+$ neurons, which express receptors for the GDNF family of neurotrophins, and $IB4^-$ neurons that predominantly express trkA.

The present inventors have shown that SNS/PN3 is expressed in approximately one-half of both $IB4^+$ and $IB4^-$ DRG neurons, while NaN is preferentially expressed in $IB4^+$ neurons. Whole-cell patch-clamp studies demonstrate that TTX-R sodium currents in $IB4^+$ neurons have a more hyperpolarized voltage-dependence of activation and inactivation than do $IB4^-$ neurons, suggesting different electrophysiological properties for SNS/PN3 and NaN. Utilizing an *in vitro* model of axotomy, it was confirmed that NGF restores SNS/PN3 mRNA levels and demonstrate that the trk antagonist K252a blocks this rescue, indicating a central role for trkA receptors in the signaling pathway. The down-regulation of NaN mRNA is, nevertheless, not rescued by NGF-treatment in either $IB4^+$ or $IB4^-$ neurons and NGF-treatment does not significantly increase the peak amplitude of the TTX-R current in small DRG neurons *in vitro*. In contrast, GDNF-treatment causes a two-fold increase in the peak amplitude of TTX-R sodium currents and restores both SNS/PN3 and NaN mRNA to near-normal levels in $IB4^+$ neurons. These observations provide a mechanism for the partial restoration of TTX-R sodium currents by NGF in axotomized DRG neurons, and demonstrate that the neurotrophins NGF and GDNF differentially regulate sodium channels SNS/PN3 and NaN.

A. TTX-R sodium channels in $IB4^+$ and $IB4^-$ DRG neurons

Several groups have suggested that DRG neurons exhibit at least two distinct types of TTX-R currents based on the voltage-dependence of activation and inactivation (Brau and Elliott, 1998; Rush et al., 1998; Scholz et al., 1998). The present inventors' results

indicate that NaN and SNS/PN3 could underlie these distinct TTX-R currents in small DRG neurons. Expression of SNS/PN3 in *Xenopus* oocytes gives rise to voltage-gated sodium currents with slow kinetics and resistance to high concentrations of TTX (Akopian et al., 1996; Sangameswaran et al., 1996). NaN, while not as yet heterologously expressed, is predicted to be TTX-R based on sequence analysis (Dib-Hajj et al., 1998b). Whole-cell patch-clamp recordings of $IB4^+$ and $IB4^-$ neurons revealed that the TTX-R currents show a voltage-dependence of steady-state activation and inactivation that is hyperpolarized in $IB4^+$ neurons. While two-thirds of $IB4^-$ cells express TTX-R currents, only about a third express NaN mRNA. Thus, SNS/PN3 may underlie much of the slow TTX-R current that is observed in $IB4^-$ cells. In support of this speculation, SNS/PN3 channels expressed in *Xenopus* oocytes give rise to slow TTX-R currents with a midpoint of inactivation of -30 mV (Akopian et al., 1996), which is similar to what it was observed in the majority of $IB4^-$ cells. Conversely, although all $IB4^+$ neurons display slow TTX-R currents, only about one half express SNS/PN3 mRNA. Since NaN is expressed predominantly in $IB4^+$ neurons, NaN may account for much of the slow TTX-R current that is observed in $IB4^+$ cells. Based on these observations, it is not unreasonable to conclude that NaN corresponds to a TTX-R sodium channel with a more negative midpoint of inactivation than SNS/PN3 and a lower threshold for activation.

The present inventors' observations that NaN is expressed in about 70% of small $IB4^+$ neurons, and that SNS/PN3 mRNA is present in slightly more than 50% of both $IB4^+$ and $IB4^-$ small neurons, indicates that NaN and SNS/PN3 are coexpressed in some of the cells. It would be predicted, therefore, that some neurons would express ensembles of NaN and SNS/PN3 currents and hence the electrophysiological analysis of $IB4^+$ and $IB4^-$ cells would underestimate the difference between the two channels. Coexpression of two slow TTX-R channels with subtly different voltage dependencies could in part account for the interneuronal variation that has been described for slow TTX-R currents in small DRG neurons (Rizzo et al., 1994). Functionally, nociceptive neurons might fine-tune their integrative and repetitive firing properties by altering the relative expression of SNS/PN3 and NaN channels. These channels may also differ in other properties, such as subcellular localization and sensitivity to second messenger modulation, which could also be important determinants of transductive and/or encoding characteristics of different DRG

neurons.

Most IB4$^+$ neurons expressed slow TTX-R currents that were similar to the TTX-R2 currents described by Rush et al. (1998); on the other hand, the TTX-R1 currents were similar to the predominant TTX-R current in IB4$^-$ cells. Fast TTX-R currents such as those described by Scholz et al. (1998) in either IB4$^+$ or IB4$^-$ cells were not observed. In the IB4$^-$ group, about one-third of the small neurons had very low amplitude, or no TTX-R currents, suggesting that this group of neurons expressed neither SNS/PN3 nor NaN. This subpopulation of IB4$^-$ neurons, which expressed relatively large fast TTX-S currents, may represent a distinct subset of sensory neurons.

B. Effect of neurotrophins on SNS/PN3 and NaN mRNA and TTX-R sodium currents

NGF has previously been shown to play a prominent role in the regulation of sodium channel/current expression in PC12 cells, as well as DRG neurons. In PC12 cells, NGF up-regulates sodium channels II and PN1 through distinct signal transduction pathways, with the latter being Ras-independent (D'Arcangelo et al., 1993). Interestingly, short-term (1-minute) application of NGF up-regulates PN1 but not brain type II in PC12 cells (Toledo-Aral et al., 1995). These observations point to divergent signaling pathways for two distinct TTX-S sodium channels (Noda et al., 1986; Klugbauer et al., 1995). Similar mechanisms may regulate specific sodium channel isoforms in DRG neurons. Consistent with this idea, NGF application accelerates the diversity and acquisition of sodium currents in neonatal DRG neurons (Omri and Meiri, 1990) and increases the threshold for spike generation in young post-natal DRG neurons (Aguayo and White, 1992).

The role of NGF in the regulation of SNS/PN3 mRNA in DRG neurons has been the focus of several studies. Administration of exogenous NGF increases the levels of SNS/PN3 mRNA both *in vitro* and *in vivo* (Black et al., 1997; Dib-Hajj et al., 1998a; this study). Moreover, enhanced levels of tissue NGF in the receptive fields of DRG neurons in carrageenan-induced inflammation are associated with up-regulation of SNS/PN3 (Tanaka et al., 1998), while, in contrast, depleted levels of NGF *in vivo* are accompanied by a down-regulation of SNS/PN3 (Fjell et al., 1999b). These observations point to an

important modulatory role for NGF in SNS/PN3 expression. In contrast, utilizing differing methodological and model systems, Okuse et al. (1997) have provided data suggesting a more limited role for NGF in SNS/PN3 expression. However, in the present study, the present inventors have confirmed the earlier observation that NGF up-regulates SNS/PN3 to near-normal levels in an *in vitro* model of axotomy, and have extended these results to show that the action of NGF on SNS/PN3 expression is not limited solely to $IB4^-$ neurons, but can also be detected in $IB4^+$ neurons. The NGF-induced up-regulation of SNS/PN3 was blocked by the trk antagonist K252a (Kase et al., 1987), suggesting that this modulatory action is mediated through a direct effect on the DRG neurons. Since a subpopulation of $IB4^+$ neurons express trkA (Bennett et al., 1998b), the increase in SNS/PN3 mRNA seen in $IB4^+$ neurons after treatment with NGF may reflect an effect on those neurons that also express trkA. NGF, however, does not regulate the expression of all sodium channel mRNAs in DRG neurons that express TrkA receptors, as NGF had no detectable effect on NaN mRNA expression.

While GDNF has well-established roles as a potent survival factor for certain classes of neurons (Lin et al., 1993; Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995) and as a protector of neurons from injury (Tomac et al., 1995; Beck et al., 1995), the effect of this neurotrophin on the electrical properties of neurons is largely unknown. It has been previously shown that intrathecal administration of GDNF ameliorates the reduction in conduction velocity of c-type fibers that follows axotomy (Bennett et al., 1998b), although it is unclear what mechanism is responsible for the enhancement of conduction velocity. The present inventors' work demonstrate here for the first time that GDNF modulates sodium channel expression, upregulating both SNS/PN3 and NaN transcripts. The action of GDNF was substantially more pronounced in $IB4^+$ neurons than in $IB4^-$neurons. Since almost all $IB4^+$, but few $IB4^-$, neurons express receptors for the GDNF-family of neurotrophins (Bennett et al., 1998b), these findings are consistent with an action of GDNF only on those neurons that express the receptor/transducer complex for GDNF.

NGF and GDNF share several characteristics as target-derived, retrogradely-transported neurotrophic factors. In the adult, NGF is primarily expressed in the skin; the levels of NGF are increased in inflamed tissues, and both endogenous and exogenous

increase of tissue NGF levels are associated with pain/hyperalgesia (Lewin et al., 1994; Woolf, 1996; Woolf et al., 1996; Dyck et al., 1997). Conversely, NGF-deprivation *in vivo* prevents hypersensitivity and results in thermal hypoalgesia (Chudler et al., 1997; Bennett et al., 1998a). In contrast to NGF, GDNF is produced at very low levels in adult skin and spinal cord, but is expressed by Schwann cells in the sciatic nerve (Nosrat et al., 1996; Widenfalk et al., 1997), suggesting that Schwann cells may be the primary source of GDNF for adult DRG neurons. It has been suggested that an up-regulation of GDNF in the injured nerve may play an important role for regeneration of sensory neurons following axotomy (Trupp et al., 1995; Hammarberg et al., 1996; Naveilhan et al., 1997; Bär et al., 1998;). Unlike the pain-inducing effect of NGF, McMahon and coworkers have suggested that GDNF may not induce pain (Bennett et al., 1998b). This hypothesis is intriguing in light of the observations of divergent central projections of $IB4^+$ and $IB4^-$ neurons (Molliver et al., 1995), and the suggestion that the former neurons are critically important in neuropathic pain and the latter in inflammatory pain (see Snider and McMahon 1998). The finding that NGF increases the expression of SNS/PN3, but not NaN mRNA, might suggest that some inflammatory syndromes could arise from an imbalance in sodium channels reflecting different responses to different neurotrophic factors. As noted above, SNS/PN3 may encode a TTX-R sodium current with a more depolarized inactivation curve and a higher threshold for activation. Changes in the levels of NGF and GDNF might therefore affect the electrogenic properties of some small DRG neurons after nerve injury, possibly contributing to the development of hyperexcitibility.

C. Methods of screening for agents to alter or modulate Sodium channel expression or activity.

Several approaches can be used to identify agents that are able to alter or modulate the GDNF induced Na^+ current through the SNS/PN3, NaN or other sodium channels. As used herein, "alter" refers to up- or down-regulating the levels or activity of NaN, such as current flow. In general, to identify such agents, a model cultured cell line that expresses the NaN sodium channel and GDNF or a GDNF receptor is utilized, and one or more conventional assays are used to measure Na^+ current. Such conventional assays include, for example, patch clamp methods, the ratiometric imaging of $[Na^+]_i$, and the use of ^{22}Na

and ^{86}Rb. Alternatively, the amount of NaN RNA or protein may be directly measured by conventional assays such as hybridization of immunoblot assays.

In one embodiment of the present invention, to evaluate the activity of a candidate compound to modulate Na^+ current, an agent is brought into contact with a suitable transformed host cell that expresses a functional GDNF receptor and NaN or GDNF. Cells that express endogenous NaN are also useful for screening of candidate agents. After mixing or appropriate incubation time, the Na^+ current is measured to determine if the agent inhibited or enhanced Na^+ current flow. If the cell line is engineered to express a functional GDNF receptor, the agent to be tested may be brought into contact with a suitable host cell in the presence or absence of exogenously supplied GDNF. Agents that inhibit or enhance Na^+ current are thereby identified.

The preferred agents that alter or modulate the levels or activity of NaN preferably will be selective for the NaN Na^+ channel, may be selective for GDNF or may alter or modulate the GDNF mediated induction of NaN. Similar analyses may be conducted by the skilled artisan to identify agents that alter the effect of other neurotrophic factors. For example, Mildbrandt, J. *et al.*. (Neuron, vol. 20, 245-253) describes the discovery of a third member of the GDNF family, Persephin, and reviews the literature on this family. The other two members are: GDNF (the prototype) and nurturin (NTN). Four receptors for these ligands have been identified. See also the March 29th, 1996 issue of the journal: Philosophical Transactions of the Royal Society of London, B. Biological Sciences (philos. trans. R. Soc. Lond. B. Biol. Sci. 1996) which contains multiple chapters on the NGF family of neurotrophic factors and their role in various models.

Agents of the invention may be totally specific (like tetrodotoxin, TTX, which inhibits sodium channels but does not bind to or directly effect any other channels or receptors), or relatively specific (such as lidocaine which binds to and blocks several types of ion channels, but has a preference for sodium channels). Total specificity is not required for an inhibitor or enhancer to be effective; the ratio of its effect on sodium channels vs. other channels and receptors, will determine its effect; and effects on several channels, in addition to the targeted one, may be of interest.

It is contemplated that modulating agents of the present invention can be, as

examples, peptides, small molecules, naturally occurring or synthetic toxins and vitamin derivatives, as well as carbohydrates. A skilled artisan will readily recognize that there is no limit as to the structural nature of the modulating agents of the present invention. It is contemplated that the screening of libraries of molecules will reveal agents that modulate NaN or current flow through it. One class of agents of the present invention are peptide agents whose amino acid sequences are chosen based on the amino acid sequence of the of the NaN Na^+ channel, of GDNF or a functional GDNF receptor. Such peptide fragments can be routinely identified by exposing a transformed host cell to these agents and measuring any resultant changes in Na^+ current. Similarly, naturally occurring toxins (such as those produced by certain fish, amphibians and invertebrates) can be screened.

D. Methods of treating pain, paraesthesia or hyperexcitability phenomena.

Agents of the invention may be administered to a human or animal subject. As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by the alteration or modulation of sodium channels such as NaN. The term "mammal" is meant an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, alteration or the modulation of the amount or of a biological activity of NaN may be associated with pain, paraesthesia or hyperexcitability phenomena. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, pain, paraesthesia or hyperexcitability phenomena may be prevented, altered or modulated by the administration of agents which reduce, enhance or modulate in some way GDNF induction of NaN.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention, such as GDNF, a GDNF-related molecule such as a GDNF peptide, or a NaN peptide can be administered in combination with another agent that alters or modulates Na^+ current. As used herein, two agents are said to be administered in

combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The present invention further provides compositions containing one or more agents which modulate expression or at least one activity of a protein of the invention. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.1 to 100 μg/kg body wt. The preferred dosages comprise 0.1 to 10 μg/kg body wt. The most preferred dosages comprise 0.1 to 1 μg/kg body wt.

In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules,

pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

GENERAL METHODS

1. *Cell culture*

Adult female Sprague-Dawley rats were deeply anesthetized with xylazine/ketamine (40/2.5 mg/kg; i.p.) and decapitated. The L4 and L5 ganglia were quickly removed and desheathed in sterile complete saline solution (CSS) (pH 7.2). The DRGs were then enzymatically digested for 20 min with collagenase A (1mg/ml; Boerhinger-Mannheim, Indianapolis, IN) in CSS and for 15 min. with collagenase D (1mg/ml; Boerhinger-Mannheim) containing papain (30 units/ml, Worthington Biochemical Corporation, Lakewood, NJ) in CSS at 37°C. The DRGs were gently centrifuged (100g for 3 min.) and the pellet triturated in DRG-media (DMEM:F12, 10% FCS) containing 1mg/ml bovine-serum albumin (BSA, Fraction V) (Sigma, St. Louis, MO) and 1 mg/ml trypsin inhibitor (Sigma). The cells were then plated on poly-ornithine/ laminin-coated glass coverslips and incubated at 37° C in a humidified 95% air /5% CO_2 incubator.

2. *Growth factors*

To study the effects of GDNF, NGF and brain-derived neurotrophic factor (BDNF) on SNS/PN3 and NaN mRNA expression, and TTX-R sodium currents, cells were treated with DRG media or DRG media supplemented with NGF (50 ng/ml, mouse 7S NGF, Sigma), GDNF (human recombinant, 50 ng/ml, Calbiochem, San Diego, CA), BDNF

(10ng/ml, Regeneron) or DRG media supplemented with a combination of NGF (50 ng/ml) and GDNF (50 ng/ml). The cells were maintained in culture for seven days and one-half of the media was replaced daily. For each experiment a control DRG culture containing neurons derived from L4/L5 ganglia was established and maintained for one day *in vitro* (1 DIV). In some experiments the trk inhibitor K252a was used to determine if the effect of NGF on SNS/PN3 mRNA expression is mediated through the trkA pathway. K252a (Calbiochem) was dissolved in DMSO (1mg/ml) and added to cultures in concentrations ranging from 100 nM - 400 nM.

3. In situ hybridization

The expression of SNS/PN3 and NaN mRNA in individual neurons was determined by *in situ* hybridization as previously described (Black et al., 1996; Dib-Hajj et al., 1998b). In short, coverslips from the different experimental groups were fixed for 10 min. in 4% formaldehyde in 0.14M Sorensons buffer, pH 7.2, washed several times with diethylpyrocarbonate (DEPC) -treated PBS and permeabilized with 0.1 % Triton X-100 in PBS for 15 min. The coverslips were then rinsed with 2X SSC, prehybridized for 30 min and then hybridized at 58°C overnight using riboprobes (0.25-0.5 ng/ml) specific for SNS/PN3 or NaN. The coverslips were sequentially incubated in 4X SSC, 2X SSC, RNase A (20mg/ml; Sigma; 37°C, 30 min.) and finally 0.2X SSC at 58°C for 3 X 20 min. The coverslips were then blocked with 2% normal sheep serum and 1 % BSA for 20 min and incubated with alkaline phosphates-conjugated anti-digoxigenin F'ab fragments (1:500, Boerhinger-Mannheim) overnight at 4°C. Following multiple rinses, the hybridization signal was visualized using NBT histochemistry. Coverslips for each condition in each experiment were kept in the NBT solution for the same length of time; the NBT reaction was monitored visually and stopped before the signal reached saturation.

For co-localization of SNS/PN3 or NaN mRNA with IB4 reactivity, biotin labeled-isolectin B4 (IB4) (40µg/ml, Sigma) was added to the culture medium and incubated for 30 min. at 37°C prior to beginning the *in situ* hybridization protocol. The coverslips were then washed with CSS and *in situ* hybridization was performed as described above with minor modifications. Following hybridization and stringent washes, the coverslips were incubated with streptavidin-Cy2 (40µg/ml, Amersham Life Science Inc, Arlington

Heights, IL) and alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500, Boerhinger-Mannheim) in Tris-buffered blocking solution (1% BSA, 2% normal goat serum) overnight at 4°C.

4. *Quantification and data analysis*

The coverslips were examined with a BioRad MRC-600 confocal microscope equipped with brightfield and BHS filter or with a Leica Aristoplan microscope. IB4 reactivity was determined visually by the presence of extracellular fluorescent signal above background levels. Microdensitometric quantification of the SNS/PN3 and NaN hybridization signal was performed as previously described (Black et al., 1997). Briefly, optical density (OD) measurements of the neurons were obtained using the Scion image analysis program. The brightfield gray levels were linearly (R^2 >0.99) calibrated to optical density using optical filters with OD = 0.1, 0.3 and 0.6. All hybridization signals measured were within the linear calibration range. Samples for analysis were obtained from each coverslip by arbitrarily scrolling the coverslip from the upper left quadrant and capturing the first twenty to fifty fields containing distinguishable neurons. The neurons in the captured images were outlined and the area and mean optical density of each cell was determined. To permit pooling of data from different experiments the optical densities were normalized by dividing the OD of each neuron by the mean SNS/PN3 or NaN optical density of the control cells at 1 DIV, processed in the same *in situ* hybridization experiment. In experiments that colocalized SNS/PN3 or NaN mRNA with IB4 binding, the OD of each $IB4^+$ or $IB4^-$ neuron was normalized as described above using the mean optical density of all ($IB4^+$ and $IB4^-$) neurons. Neurons were considered positive for SNS/PN3 or NaN if the relative intensity was > 0.8, which corresponds to a lightly stained neuron. In experiments with K252a added to the medium, the mean OD of all neurons from cultures that had not received any supplement was used to normalize the experimental values.

The Mann-Whitney Rank sum test was used to test if statistically significant differences exist in the expression of SNS/PN3 and NaN mRNA in $IB4^+$ and $IB4^-$ neurons. To determine if the effects of the different neurotrophins on SNS/PN3 and NaN mRNA expression in $IB4^+$ and $IB4^-$ neurons were statistically significant, the means of the normalized optical densities pooled from 3 separate experiments were analyzed using a

one-way ANOVA. If a significant difference was detected, a two-tailed Student *t* test was used and the resulting p-value was corrected by multiplying by the number of comparisons made (Bonferroni *t* test). Significance was assessed as $p_{\text{corrected}}$ (p_c) < 0.05.

5. Whole-cell Recordings

Sodium currents were recorded from DRG neurons in the whole-cell patch-clamp configuration 18-30 hrs. after dissociation and plating (1DIV) or after treatment with growth factors for seven days *in vitro* (7 DIV). Prior to recording, the cells were incubated for 30-60 minutes with FITC-labeled Isolectin B4 (40μg/ml, Sigma). All recordings were made with an EPC-9 amplifier, a Macintosh Quadra 950 and the Pulse program (v 7.52, HEKA Electronic, Germany). Recording electrodes (0.8-2 MΩ) were fabricated from 1.65-mm capillary glass (WPI) using a Sutter P-87 puller. Cells were not considered for analysis if the initial seal resistance was less than 1 GΩ or if they had high leakage currents (holding current > 1 nA at - 80 mV) or an access resistance greater than 5 MΩ. The average access resistance was 2.3 ± 0.8 MΩ (mean ± standard deviation, n=310). Voltage errors were minimized using 70-80% series resistance compensation. Linear leak subtraction and capacitance artifact cancellation were used for all recordings. Membrane currents were filtered at 2.5 KHz and sampled at 10 KHz. The pipette solution contained (in mM): 140 CsF, 2 $MgCl_2$, 1 EGTA, and 10 Na-HEPES (pH 7.3). The standard extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 0.1 $CdCl_2$, and 10 HEPES (pH 7.3). Cadmium was included to block calcium currents. The osmolarity of the solutions was adjusted to 310 mosM (Wescor 5550 osmometer). The liquid junction potential for these solutions was <7 mV; data were not corrected to account for this offset. The offset potential was zeroed before patching the cells and checked after each recording for drift. All recordings were conducted at room temperature (~22° C).

Example 1: ***SNS/PN3 and NaN mRNA in IB4$^+$ and IB4$^-$ DRG neurons***

Both SNS/PN3 and NaN mRNA are suggested to encode TTX-R sodium channels in DRG neurons (Akopian et al., 1996; Sangameswaran et al., 1996; Dib-Hajj et al.,

1998b), and both are preferentially expressed in small (< 30 μm) DRG neurons (Fig. 1). Small DRG neurons have been differentiated into two major subpopulations on the basis of their ability to bind to the lectin IB4 (Averill et al., 1995; Wright and Snider, 1995; Molliver et al., 1997; Bennett et al., 1998b). To establish if SNS/PN3 and NaN are differentially expressed in $IB4^+$ and $IB4^-$ neurons, localization of SNS/PN3 or NaN mRNA by *in situ* hybridization was combined with IB4 cytofluorescent labeling. IB4 binding was determined by the presence of a clearly identifiable extracellullar staining of the soma (Fig. 2). SNS/PN3 mRNA was expressed in both $IB4^+$ and $IB4^-$ neurons, but neurons with the greatest hybridization signal for SNS/PN3 mRNA were predominantly $IB4^-$. The difference in SNS/PN3 mRNA expression between $IB4^+$ and $IB4^-$ neurons was significant ($p < 0.05$, Mann-Whitney rank sum test) (Fig. 3a). Fifty-two percent of small $IB4^+$ neurons (n=157) and 64% of small $IB4^-$ neurons (n=226) expressed SNS/PN3 mRNA. In contrast to SNS/PN3, NaN was expressed predominantly in $IB4^+$ neurons ($p < 0.0001$, Mann-Whitney rank sum test) (Fig. 3b.), with 72% of small $IB4^+$ neurons (n=144) and only 38% of small $IB4^-$ neurons (n=98) expressing NaN mRNA. These results demonstrate that SNS/PN3 and NaN are differentially expressed in $IB4^+$ and $IB4^-$ DRG neurons; moreover, the distribution patterns indicate that SNS/PN3 and NaN must be coexpressed in a substantial subpopulation of $IB4^+$ DRG neurons, and possibly some $IB4^-$ neurons.

Example 2: ***TTX-R currents in $IB4^+$ and $IB4^-$ neurons***

Since SNS/PN3 and NaN have different patterns of expression in small $IB4^+$ and $IB4^-$ neurons, whole-cell patch-clamp recordings from small $IB4^+$ and $IB4^-$ (17-32 μm diam.) DRG neurons were performed to determine if differences exist in voltage-dependence and kinetic properties of sodium currents in these cells. The neurons were maintained in culture for less than 30 hrs and binding to IB4 was determined prior to recording, permitting us to analyze approximately the same number of $IB4^+$ and $IB4^-$ neurons. Both fast inactivating ("fast", $\tau_h < 1$ msec at 0 mV) and slow inactivating ("slow", $\tau_h > 2.5$ msec at 0 mV) sodium currents were observed in small DRG neurons. The fast and slow sodium currents observed in the present study were similar to those previously described in small DRG neurons (Caffrey et al., 1992; Roy and Narahashi,

1992; Elliott and Elliott, 1993; Rizzo et al., 1994; Cummins and Waxman, 1997). All $IB4^+$ neurons (n=33) analyzed expressed slow sodium currents, and all but one of these $IB4^+$ cells also expressed fast sodium currents. In contrast, only 63% of the $IB4^-$ neurons (n=32) expressed slow currents, but all $IB4^-$ neurons expressed fast currents. Based on these observations, neurons were assigned to one of three groups: $IB4^+$ neurons with both fast and slow currents ($IB4^+$ F/S), $IB4^-$ neurons with both fast and slow currents ($IB4^-$ F/S), and $IB4^-$ neurons with only fast currents ($IB4^-$ F) (Fig. 4A).

Prepulse-inactivation was used (McLean et al., 1988; Roy and Narahashi, 1992; Elliott and Elliott, 1993; Cummins and Waxman, 1997) to separate fast and slow sodium current components in the cells. The peak amplitudes for fast and slow sodium current components are compared in Table 1. The slow current peak amplitude was similar for $IB4^+$ F/S and $IB4^-$ F/S cells, but the fast current amplitude was smaller for $IB4^+$ F/S cells than for the $IB4^-$ F/S cells. Because previous studies have shown that the fast current is TTX-sensitive (TTX-S) and the slow current is TTX-R, and because both SNS/PN3 and NaN putatively underlie TTX-R currents in DRG neurons, the properties of the slow currents in $IB4^+$ and $IB4^-$ cells were examined. The mean midpoints of activation and steady-state inactivation for slow currents were more negative for $IB4^+$ than for $IB4^-$ cells (Fig. 4C). While these differences are small, they are significant ($p < 0.005$). Figure 4B shows the distribution of inactivation midpoint values in individual $IB4^+$ and $IB4^-$ cells.

Table 1. Current amplitude: comparison of sodium currents in IB4$^+$ and IB4$^-$ neurons.

	IB4$^+$		IB4$^-$	
	with slow	w/o slow	with slow	w/o slow
0 nM TTX				
fast amplitude (nA)	29±2	---	46±10	31±4
slow amplitude (nA)	31±3	---	32±8	0.7±0.3
% cells	100%	0%	63%	37%
250 nM TTX				
TTX-R amplitude (nA)	38±4	---	37±7	1.0±0.2
% cells	100%	0%	60%	40%

Although the present inventors have previously shown that prepulse subtraction and TTX-subtraction give essentially the same results (Cummins and Waxman, 1997), indicating that all of the slow current is TTX-R and all of the fast current is TTX-S in DRG neurons, Scholz et al. (1998) observed a fast TTX-R current in young DRG neurons (7-21 days), raising the possibility that SNS/PN3 or NaN might encode a fast TTX-R current in DRG neurons. Therefore, sodium currents in $IB4^+$ and $IB4^-$ neurons in the presence of 250 nM TTX, which blocks 98% of the TTX-S current were also examined. Under these conditions, all $IB4^+$ cells (n=30) but only 60% of $IB4^-$ neurons (n=30) expressed large (>200 pA/pF) sodium currents. Thus, in agreement with the present inventors' prior classification, DRG neurons studied in the presence of 250 nM TTX can also be subdivided into three groups: $IB4^+$ with TTX-R sodium current, $IB4^-$ with TTX-R sodium current and $IB4^-$ without TTX-R sodium current (Fig. 5A, Table 1). The amplitude of the TTX-R current was similar for the $IB4^+$ and $IB4^-$ groups that expressed sodium currents.

The rate of inactivation (τ_h) for the TTX-R sodium currents in the $IB4^+$ and $IB4^-$ cells was compared. τ_h, measured at 0 mV, was significantly ($p < 0.05$) slower for the currents in $IB4^-$ cells (6.1 ±0.7 ms, n=18) than for the currents in $IB4^+$ cells (4.6 ±0.3 ms, n=30). However, while τ_h was longer in $IB4^-$ cells than in $IB4^+$ cells, all of the TTX-R currents in both groups had time constants greater than 2.5 ms and therefore are considered slow currents. Since all of the currents recorded in the presence of TTX were slow, and since fast TTX-R sodium currents were not observed in either $IB4^+$ cells or $IB4^-$ cells, the present inventors' data suggest that both NaN and SNS/PN3 encode slow TTX-R currents. It is interesting to note that the percentage of $IB4^-$ cells that expressed little or no sodium current in the presence of 250nM TTX was similar to the percentage of $IB4^-$ cells that expressed only fast sodium currents in the absence of TTX. Thus, as with previous studies (Kostyuk et al., 1981; McLean et al., 1988; Roy and Narahashi, 1992; Cummins and Waxman, 1997), the data are consistent with the fast currents being solely TTX-S and slow currents being solely TTX-R.

The midpoint of activation and steady-state inactivation of the TTX-R current was significantly ($p < 0.001$) more negative for $IB4^+$ neurons than for $IB4^-$ neurons that produce TTX-R sodium currents (Fig. 5B). The midpoint of steady-state inactivation ranged from

-31 to -44 mV for TTX-R currents in IB4$^+$ cells and from -25 to –36 mV for TTX-R currents in IB4$^-$ cells. Fig. 5C shows the distribution of the midpoints of activation and steady-state inactivation for the TTX-R currents. While 50% of IB4$^+$ cells have a midpoint of inactivation that is more negative than or equal to -37 mV, none of the IB4$^-$ cells do. Conversely, while 39% of the IB4$^-$ cells have a midpoint of inactivation that is positive to -31 mV, none of the IB4$^+$ cells do. However, while the extremes were dominated by IB4$^+$ cells and IB4$^-$ cells, respectively, there was overlap between the two groups.

Example 3: ***Effect of GDNF and NGF on SNS/PN3 mRNA expression***

The effect of GDNF and NGF on SNS/PN3 mRNA expression was examined in IB4$^+$ and IB4$^-$ neurons dissociated from adult L4/L5 DRG and cultured for 7 days with normal culture media ("control"), or with media supplemented with NGF, GDNF, BDNF or a combination of NGF and GDNF. DRG neurons dissociated and cultured in this manner, in the absence of exogenously added growth factors, have previously been shown to display changes in levels of sodium channel III and SNS/PN3 mRNAs similar to those seen 7 days after nerve transection *in vivo* (Dib-Hajj et al., 1996; Black et al., 1997). In agreement with a role for GDNF in the maintenance of IB4 reactivity in a subpopulation of small DRG neurons (Bennett et al., 1998b), the IB4 signal intensity was reduced, but discernable, in neurons maintained in cultures for 7 days without exogenously-added GDNF.

In comparison to 7 DIV control neurons, GDNF treatment significantly ($p_c < 0.01$) upregulated SNS/PN3 in both IB4$^+$ and IB4$^-$ neurons (Fig. 6). The effect of GDNF on SNS/PN3 mRNA expression was significantly ($p_c < 0.001$) more prominent in IB4$^+$ neurons (n=74) than in IB4$^-$ neurons (n= 51). Similar to GDNF treatment, NGF supplement significantly ($p_c < 0.001$) enhanced the SNS/PN3 hybridization signal in both IB4$^+$ (n=91) and IB4$^-$ (n=72) neurons compared to IB4$^+$ and IB4$^-$ 7 DIV control neurons (Fig. 6). However, unlike GDNF treatment, NGF did not preferentially upregulate (p = ns) SNS/PN3 in IB4$^-$ neurons (n=91) compared to IB4$^-$ neurons (n=72) (Fig. 6, 7). The combination of NGF and GDNF significantly increased the SNS/PN3 hybridization signal

in both $IB4^+$ and $IB4^-$ neurons compared to 7 DIV control neurons; however, the signal in $IB4^+$ neurons was less than that observed with either treatment alone, while $IB4^-$ neurons showed similar hybridization signals for the neurotrophins alone or in combination (Fig. 6). In contrast, it was observed that BDNF had no effect on SNS mRNA levels.

Example 4: ***NGF up-regulation of SNS/PN3 mRNA is blocked by K252a***

The up-regulation of SNS/PN3 by NGF could be mediated through the trkA pathway; alternatively, the upregulation may be through a pathway involving the p75 receptor. K252a, in the 100-400 nM range, is a potent inhibitor of NGF action through the trk receptor and has been used to separate effects of NGF mediated through the high-affinity trkA receptor and the low affinity p75 receptor (Kase et al., 1987; Doherty and Walsh, 1989; Tapley et al., 1992; Kahle et al., 1994; Buck and Winter, 1996; De Bernardi et al., 1996). In control experiments without addition of K252a, exogenously-added NGF significantly ($p_c < 0.001$) increased the expression of SNS/PN3 mRNA in DRG neurons maintained in culture for 7 DIV compared to neurons in culture for 7 DIV without added NGF (Fig.8). K252a blocked the effect of NGF on SNS/PN3 expression in a concentration-dependent manner at all concentrations of K252a tested (100- 400nM). The addition of K252a alone did not have a significant effect on SNS/PN3 mRNA expression compared to untreated controls (Fig. 8). These observations indicate that trkA is a necessary component for the effect of NGF on the expression of SNS/PN3 mRNA. Since Schwann cells, which are present in the cultures, express p75 but not trkA (Yamamoto et al., 1993), these results argue that the effect of NGF on SNS/PN3 expression is mediated through a direct action involving TrkA on DRG neurons.

Example 5: ***Effect of GDNF and NGF on NaN mRNA***

While NaN expression is decreased following axotomy (Dib-Hajj et al., 1998b) the effect of culturing on NaN mRNA expression has not previously been established. As described for SNS, maintaining of DRG neurons in culture for 7 days without addition of growth factors (n=125) significantly ($p_c < 0.001$) reduced the levels of NaN mRNA compared to freshly dissociated neurons (1 DIV) (n= 95). The reduction was, however,

limited to IB4$^+$ neurons, as no significant change was seen in IB4$^-$ neurons.

To examine if NaN mRNA is regulated by NGF and GDNF, NaN hybridization signals of DRG neurons treated in culture for 7 days with NGF (n=99), GDNF (n=122), BDNF (n= 93) or a combination of GDNF and NGF (n= 97) were compared to those in control cultures maintained for 7 days without addition of growth factors (n=125). In contrast to the effect on SNS/PN3 mRNA, NGF addition to the culture medium did not alter NaN levels in DRG neurons at 7 DIV in either IB4$^+$ (n= 45) or IB4$^-$ (n= 54) neurons (Fig. 9,10). In contrast, GDNF supplement to the culture medium significantly ($p_c <$ 0.001) increased NaN hybridization signal in IB4$^+$ neurons (n= 83) but had no effect on IB4$^-$ neurons (n= 39) (Fig. 9,10). These results indicate that GDNF, but not NGF, regulates the expression of NaN mRNA, and are consistent with a direct effect of GDNF on sensory neurons that express receptors for the GDNF–family of neurotrophins. When combining GDNF and NGF, the NaN hybridization was significantly ($p_c < 0.001$) increased in IB4$^+$ neurons (n= 57) compared to 7 DIV control neurons, whereas no significant change was seen in IB4$^-$ (n= 40) neurons. The hybridization signal in IB4$^+$ neurons treated with the combination of GDNF and NGF was somewhat lower than in cultures treated with GDNF alone (Fig. 9). BDNF had no significant effect on the levels of NaN expression compared to control

neurons.

Example 6: ***Effect of GDNF and NGF treatment on TTX-R currents***

Sodium currents in small DRG neurons after 7 DIV in the absence (control) or presence of exogenously added NGF, GDNF or combination NGF and GDNF was examined. For these experiments, IB4-reactivity after each cell was selected for recording was also determined. The staining obtained with FITC-labeled IB4 was much less intense at 7 DIV than that observed at 1 DIV. The intensity of IB4 fluorescence was clearly greater in the GDNF and GDNF/NGF groups than it was in the control and NGF groups at 7 DIV. In the absence of exogenously added GDNF, the reduced fluorescence intensity made it difficult to accurately classify the cells as IB4$^+$ or IB4$^-$. In contrast, IB4-reactivity in the *in situ* hybridization experiments (see above) was assessed with biotin-labeled IB4

and Cy2-conjugated streptavidin, which provide for enhanced signal amplification. Because of the uncertainty in the IB4-reactivity classification in the patch-clamp experiments on DRG neurons after 7 DIV, sodium current data was not subdivided into $IB4^+$ and $IB4^-$ cell groups .

Sodium currents were recorded in the presence of 250 nM TTX to isolate TTX-R currents. Figure 11A shows representative currents for each of the four groups of neurons. In the control group the amplitude of the TTX-R current was significantly lower (6.1±1.9 nA, n=41) than that recorded at 1 DIV (23.9±12.5 nA, n=36, Fig. 11B). This reduction in TTX-R currents following *in vitro* axotomy is very similar to that observed following *in vivo* axotomy (Cummins and Waxman, 1997). In neurons treated with NGF, the TTX-R current amplitude was similar (6.2±1.5 nA, n=40) to that observed in control neurons at 7 DIV. Neurons treated with GDNF, on the other hand, had significantly larger TTX-R currents (13.7±2.4 nA; n=40) than that of control neurons at 7 DIV ($p < 0.02$; Fig. 11B). The GDNF/NGF group had the largest peak current amplitude (16.1±2.4 nA, n= 40), although this was not significantly different from that of the GDNF group. As a measure of TTX-R current expression, the percentage of cells expressing TTX-R current amplitudes > 3 nA (Table II) was determined. In contrast to neurons at 1 DIV, where ~ 90 % of cells displayed TTX-R currents > 3 nA, at 7 DIV less than a quarter of the control cells had TTX-R current amplitudes > 3 nA. GDNF treatment was far superior to NGF treatment in increasing the proportion of cells with large TTX-R currents (Table II).

Because of the extensive neurites that develop in culture, a detailed characterization of the voltage-dependent and kinetic properties of the TTX-R currents in neurons at 7 DIV was not made. However, the TTX-R currents in all four groups appeared slow and the midpoints of activation and steady-state inactivation at 7 DIV were generally similar to those observed for $IB4^+$ cells at 1 DIV.

Table II:

	1 DIV	7 DIV			
		Control	NGF	GDNF	GDNF/NGF
% cells with I_{Na} > 3 nA:	91±5%	24±7%	38±8%	60±8%	68±7%
cell capacitance:	27±2pF	30±2pF	28±2pF	33±2pF	35±2pF
number cells:	36	41	40	40	40

Example 7: ***Effect of GDNF and NGF on TTX-R currents in small DRG neurons from SNS-null mice***

Small DRG neurons were taken from SNS-null mice (Akopian et al., 1999) and cultured *in vitro* using the methods described for rat neurons above. Neurons were cultured in the presence of 250 nM TTX and further in the presence or absence of GDNF (10ng/ml) or NGF (50 ng/ml). The currents were elicited by 200 ms test pulses to potentials ranging from -80 to +40 mV in 5 mV steps. Cells were held at -100 mV. As shown in Figures 12A, in untreated cultures the persistent non-SNS TTX-R sodium current traces were drastically decreased after 7 days in vitro (7 DIV). By contrast, in GDNF-treated cultures the TTX-R currents were maintained at control levels. Similarly, persistent non-SNS TTX-R peak currents were returned to control levels by the addition of GDNF, as shown in Figure 12B. Notably, the addition of NGF to the culture medium did not significantly effect the size of persistent non-TTX-R current compared to the control 7 DIV neurons.

Example 8: ***GDNF attenuates the decrease of TTX-R current density and persistent Na^+ currents*** **in vivo**

Uninjured neurons predominantly express slow-inactivating TTX-R and slowly-repriming TTX-S Na^+ currents. Following axotomy, TTX-R current density is greatly reduced and rapidly repriming TTX-S currents predominate. Consequently, the effect *in vivo* of exogenously administered GDNF delivered to transected nerves on sodium currents in small DRG neurons was examined. GDNF (1.2 μg/day per animal) was delivered to DRG neurons *in vivo* via an osmotic pump attached to the transected sciatic nerve. (Dib-Hajj et al. 1998a). The opposite sciatic nerve was also transected and hooked to a pump containing only Ringer's solution.

As shown in Figures 13A and 13B, both slowly-inactivating and persistent TTX-R currents were partially restored to toward control levels in GDNF-treated axotomized

neurons. It was also observed that TTX-S currents in GDNF-treated axotomized neurons expressed intermediate repriming kinetics. Thus, GDNF treatment can mitigate the effects of axotomy on the sodium currents of a sub-population of small DRG sensory neurons.

Although the present invention has been described in detail with reference to the examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety.

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CLAIMS

1. A method to treat pain or hyperexcitability phenomena in an animal or human subject by administering an amount of GDNF that is effective to alter TTX-R Na^+ current flow through NaN sodium channels in DRG or trigeminal neurons.

2. The method of claim 1, wherein the sensory neuron is a DRG or trigeminal neuron.

3. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of GDNF capable of at least partially restoring the normal balance between various types of TTX-R and TTX-S sodium channels in sensory neurons.

4. The method of claim 3, wherein the sensory neuron is a DRG or trigeminal neuron.

5. The method of claim 3 or 4, wherein the TTX-R sodium channels are selected from the group consisting of SNS/PN3 and NaN channels.

6. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of modulating the transcription or translation of mRNA encoding sodium channels selected from the group consisting of SNS/PN3 and NaN channels.

7. The method of claim 6, wherein the agent is a neurotrophin.

8. The method of claim 6, wherein the neurotrophin is selected from the group

consisting of NGF and GDNF and or other members of their families.

9. The method of claim 6, wherein the agent modulates the production or activity of a neurotrophin that modulates the activity of the sodium channel.

10. The method of claim 9, wherein the agent modulates the level or activity of GDNF or NGF.

11. A method to treat pain, paraesthesia or hyperexcitability phenomena in an animal or human subject by administering an effective amount of an agent capable of altering the transcription or translation of mRNA encoding the NaN sodium channel.

12. A method of identifying an agent which modulates TTX-R Na^+ current through NaN channels comprising the step of:

determining whether the agent alters or modulates the expression of GDNF or at least one biological activity of GDNF.

13. The method of claim 10, wherein the agent modulates the GDNF induction of NaN.

14. A cell that has been transformed to express a functional recombinant GDNF receptor and, optionally, recombinant NaN.

15. A method to screen candidate compounds for use in treating pain and hyperexcitability phenomena comprising the steps of exposing the cell to the compound in the presence or absence of GDNF and determining the resultant level of expression or activity of the cell's Na^+ channels.

16. The method of claim 15, wherein the cell is the transformed cell of claim 12.

17. The method of claim 15, wherein the Na^+ channel is selected from the group consisting of the SNS/PN3 and NaN channels.

18. The method of claim 15, wherein the cell does not express SNS.

19. The method of claim 15, wherein the cell is present in a living animal.

FIG. 1A

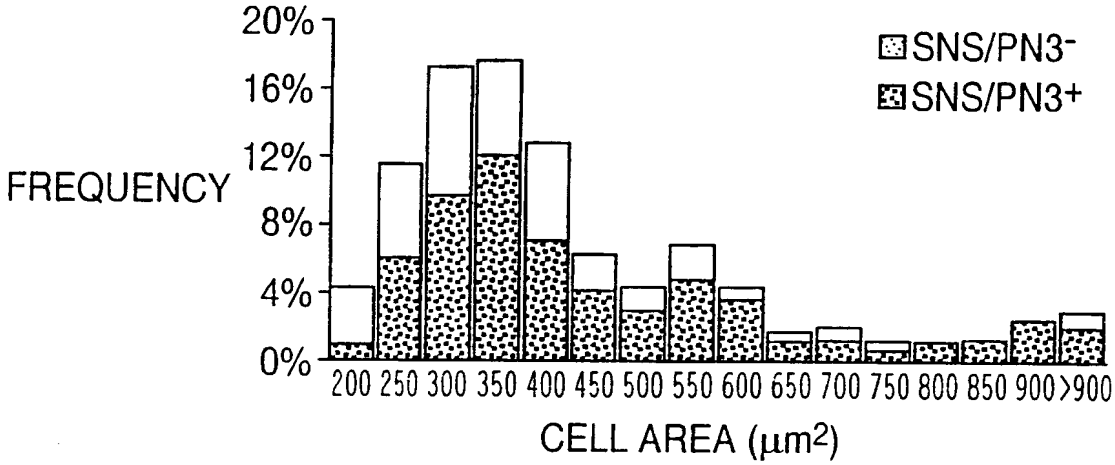

20%
16%
12%
8%
4%
0%
FREQUENCY
SNS/PN3-
SNS/PN3+
200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 >900
CELL AREA (μm2)

FIG. 1B

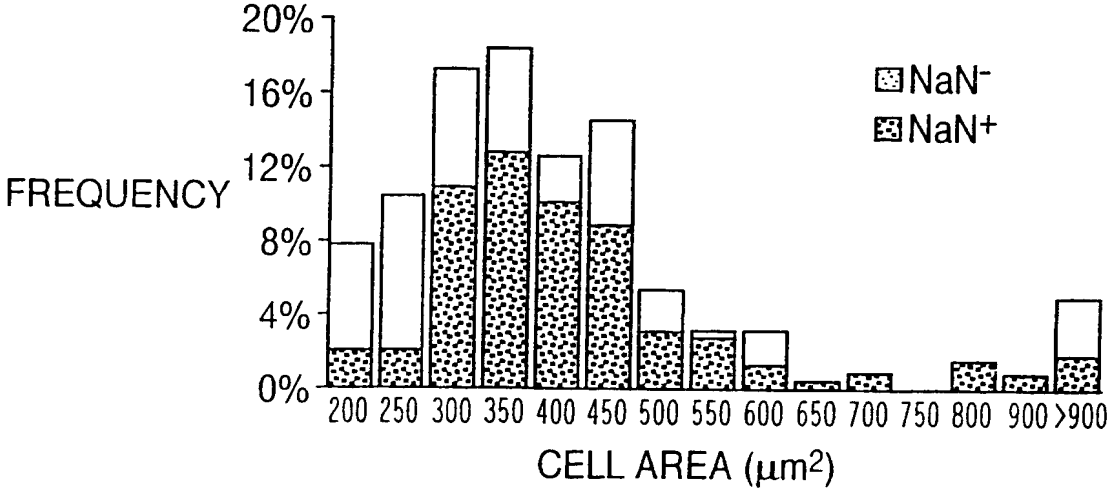
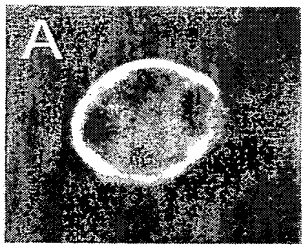

20%
16%
12%
8%
4%
0%
FREQUENCY
NaN-
NaN+
200 250 300 350 400 450 500 550 600 650 700 750 800 900 >900
CELL AREA (μm2)

FIG. 2A



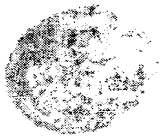


FIG. 2B

FIG. 2C

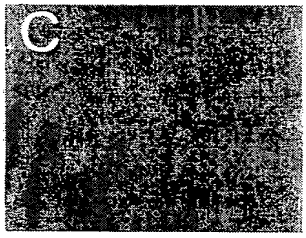
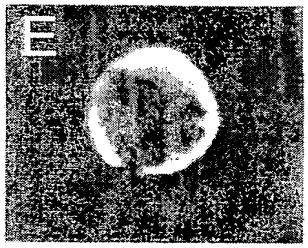


FIG. 2D

FIG. 2E



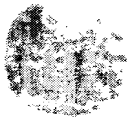


FIG. 2F

FIG. 2G

FIG. 2H

FIG. 3A

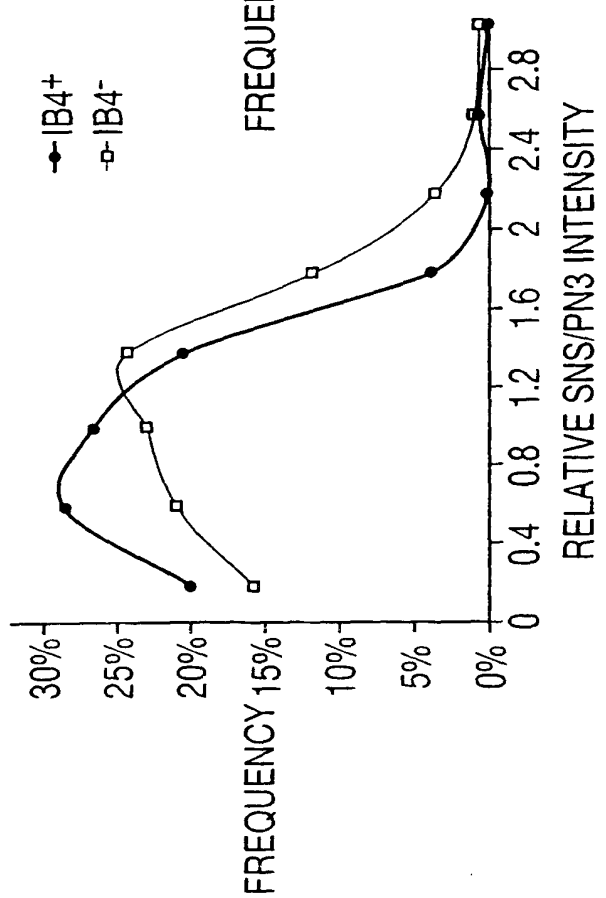

IB4+
IB4-
FREQUENCY
30%
25%
20%
15%
10%
5%
0%
0
0.4
0.8
1.2
1.6
2
2.4
2.8
RELATIVE SNS/PN3 INTENSITY

FIG. 3B

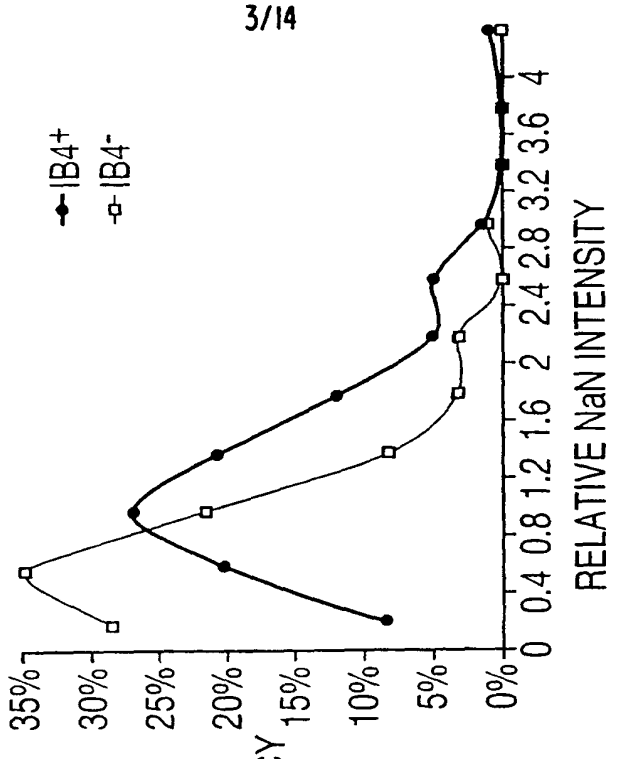

IB4+
IB4-
FREQUENCY
35%
30%
25%
20%
15%
10%
5%
0%
0
0.4
0.8
1.2
1.6
2
2.4
2.8
3.2
3.6
4
RELATIVE NaN INTENSITY

FIG. 4A

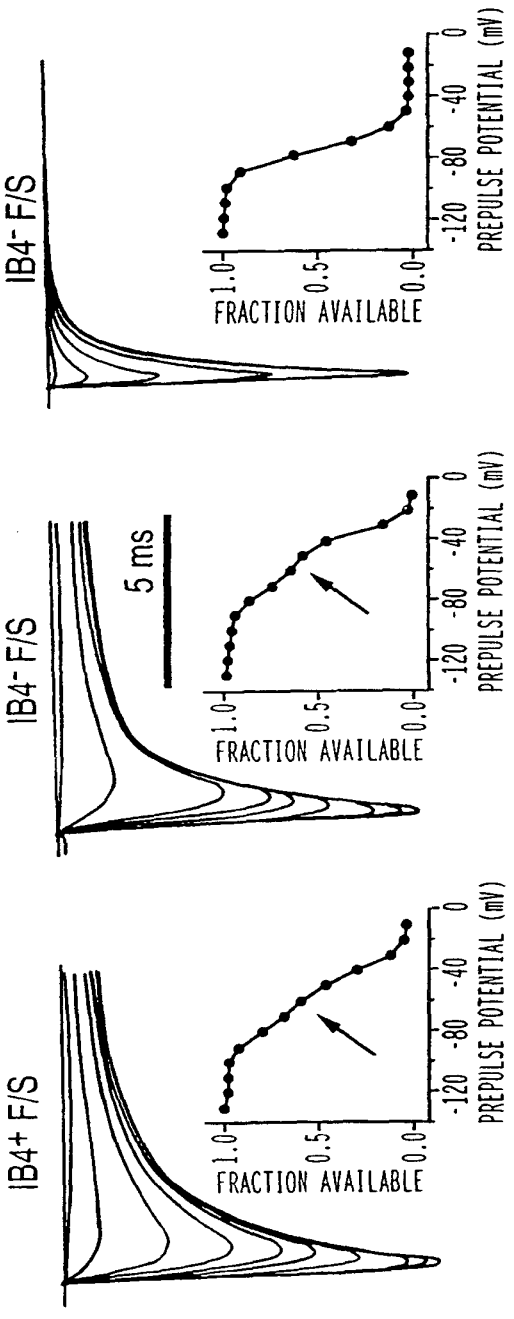


FIG. 4B

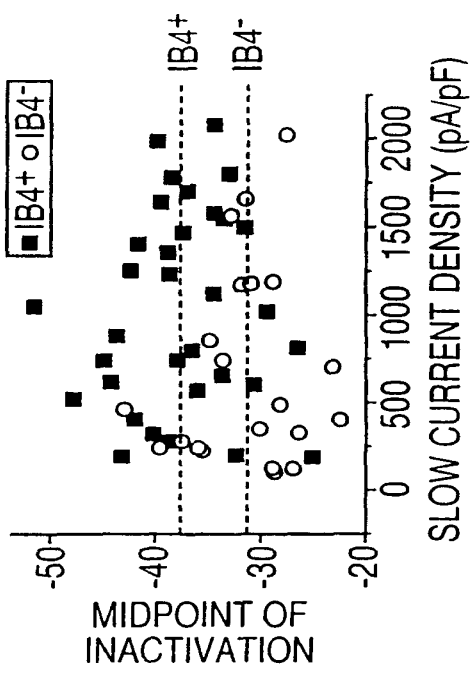
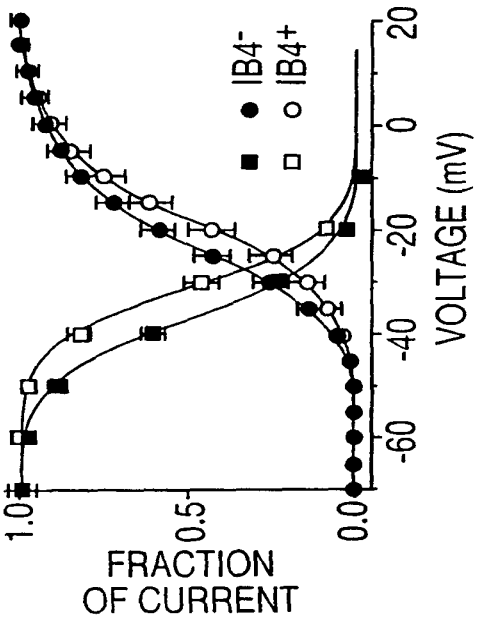


FIG. 4C



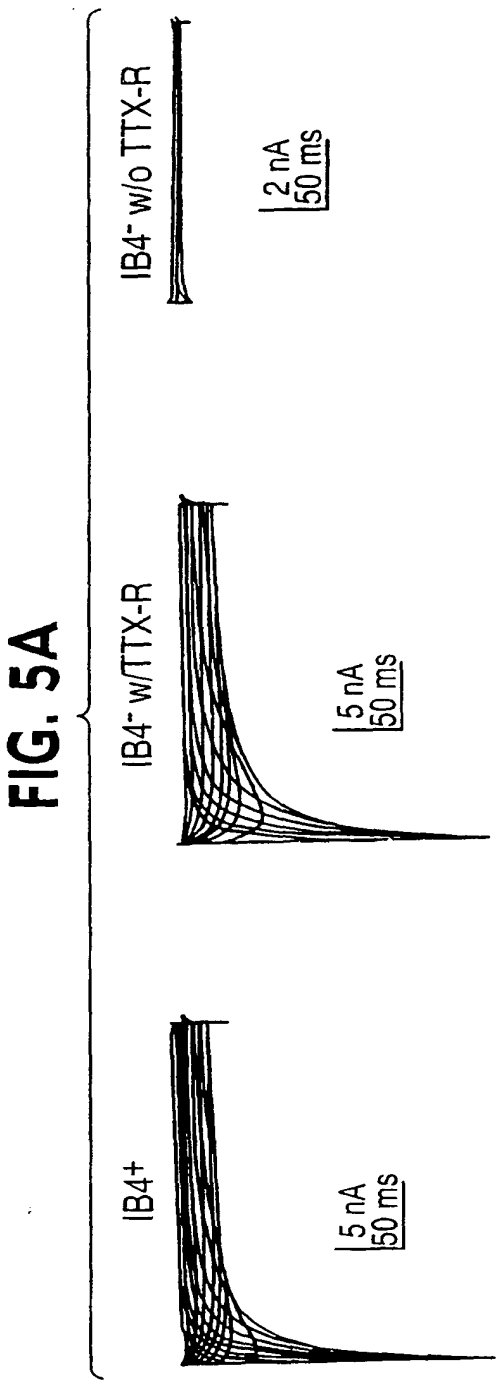


FIG. 5A

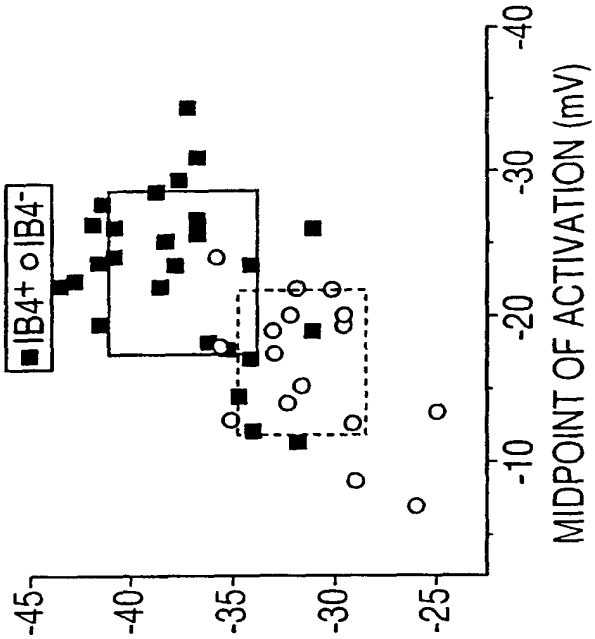


FIG. 5C

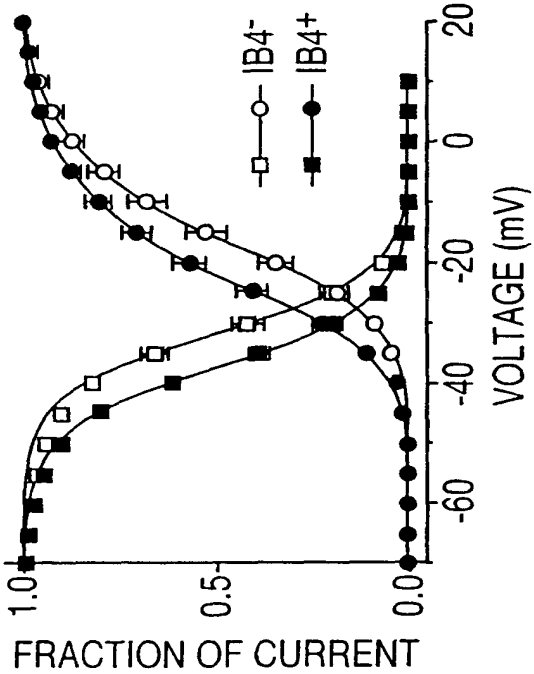


FIG. 5B

FIG. 6

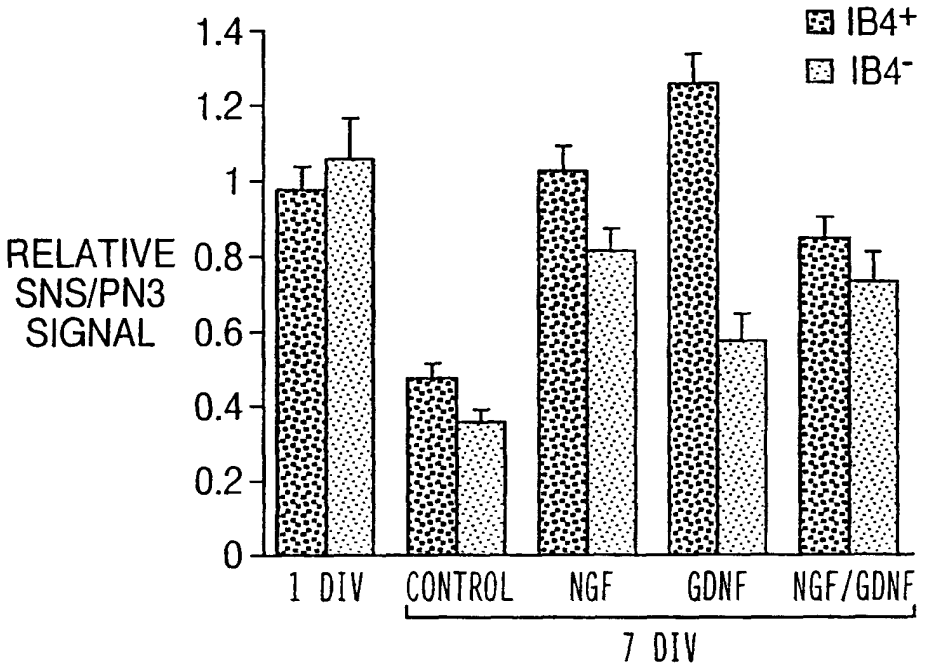

IB4+
IB4-
RELATIVE SNS/PN3 SIGNAL
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 DIV
CONTROL
NGF
GDNF
NGF/GDNF
7 DIV

FIG. 7A A

B FIG. 7B

FIG. 7C C

D

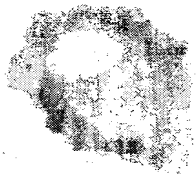


FIG. 7D

FIG. 7E E

F FIG. 7F

FIG. 8

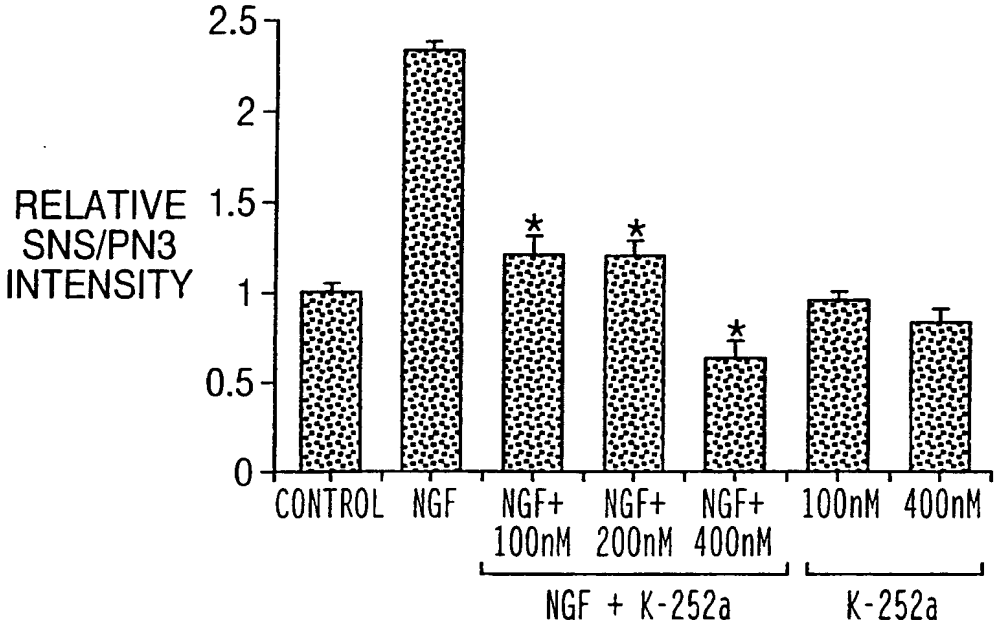

RELATIVE SNS/PN3 INTENSITY
2.5
2
1.5
1
0.5
0
CONTROL
NGF
NGF+ 100nM
NGF+ 200nM
NGF+ 400nM
100nM
400nM
NGF + K-252a
K-252a

FIG. 9

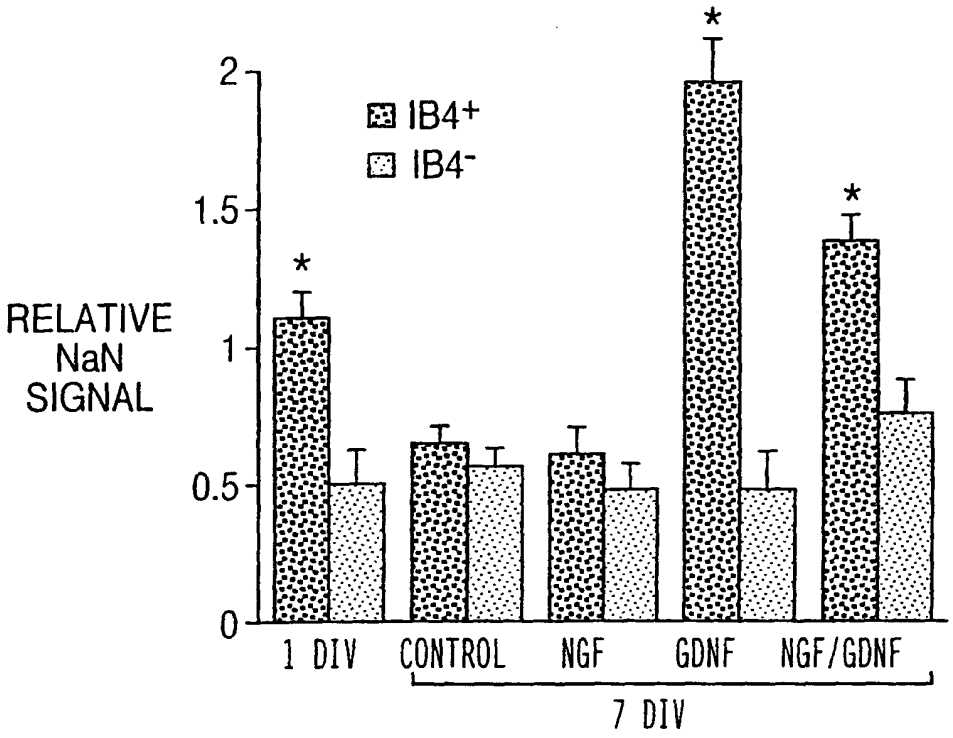

RELATIVE NaN SIGNAL
2
1.5
1
0.5
0
IB4+
IB4-
*
*
*
1 DIV
CONTROL
NGF
GDNF
NGF/GDNF
7 DIV

FIG. 10A

FIG. 10B

FIG. 10C

FIG. 10D

FIG. 10E

FIG. 10F

FIG. 11A

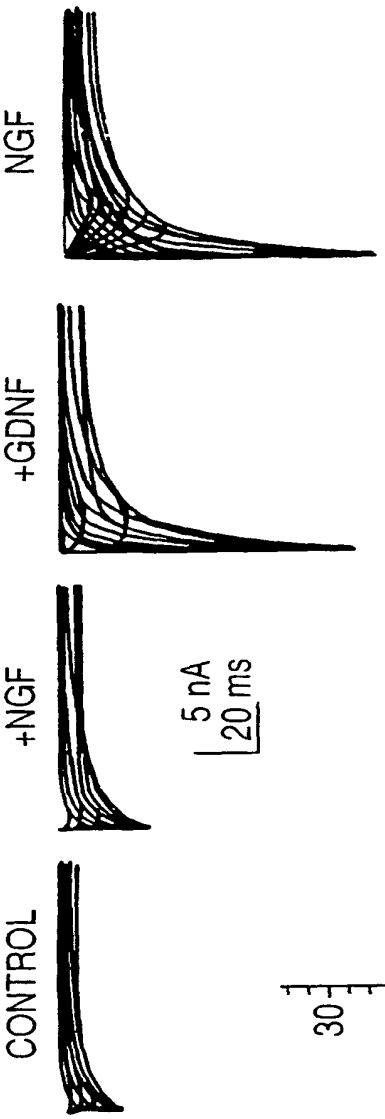
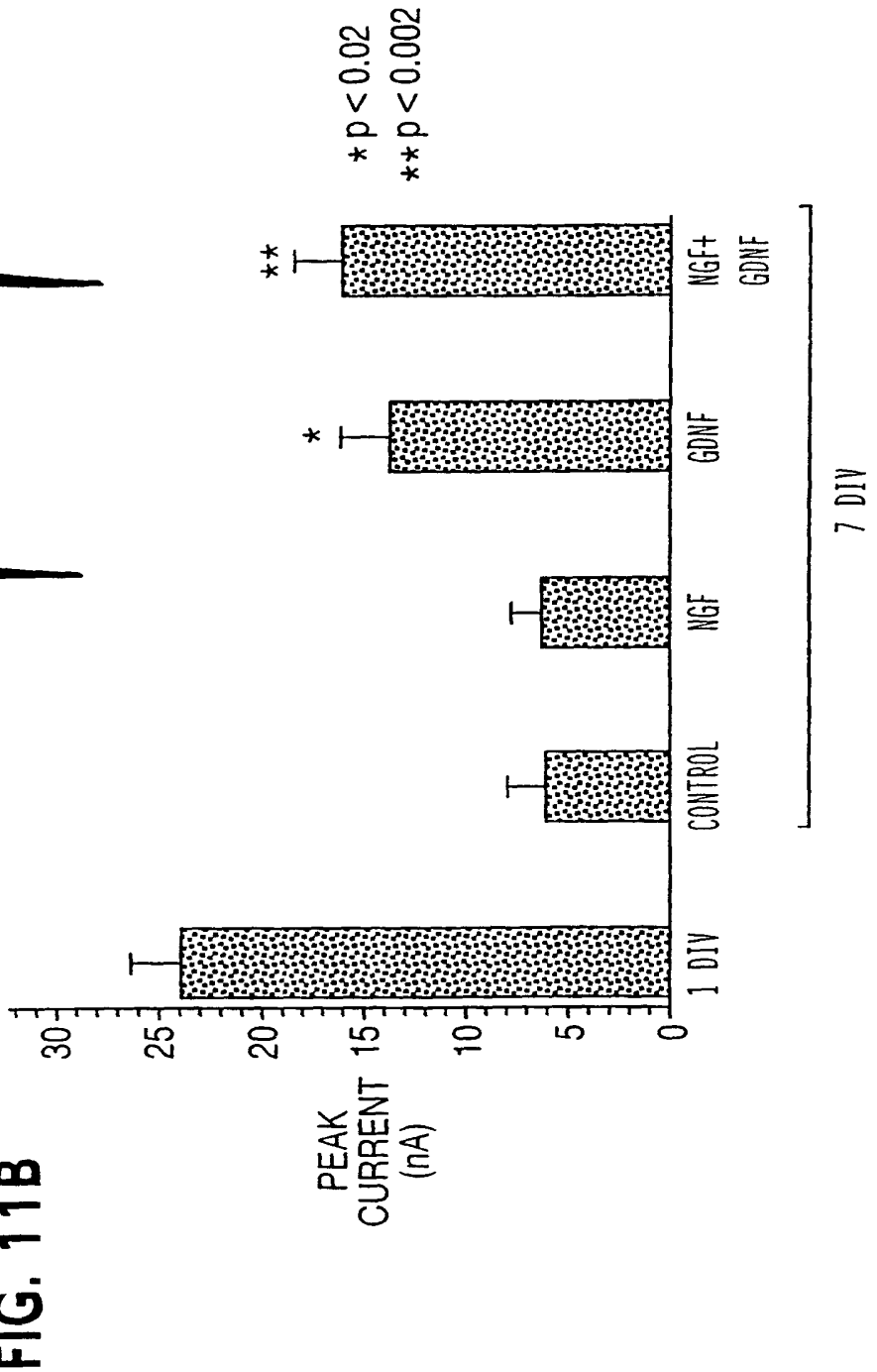


FIG. 11B



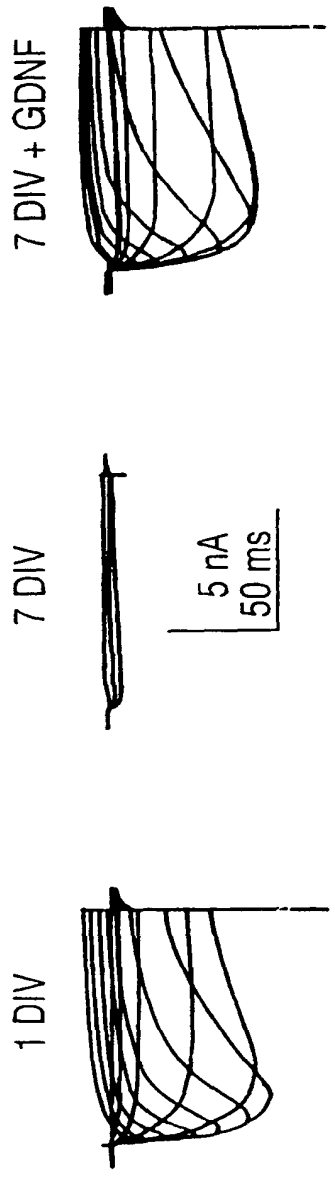


FIG. 12A

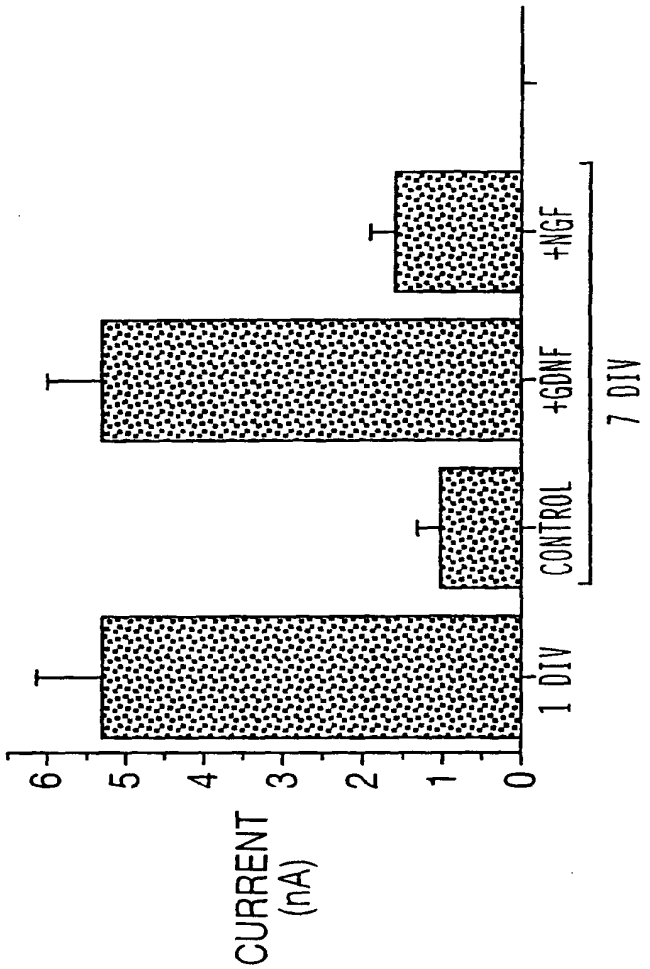


FIG. 12B

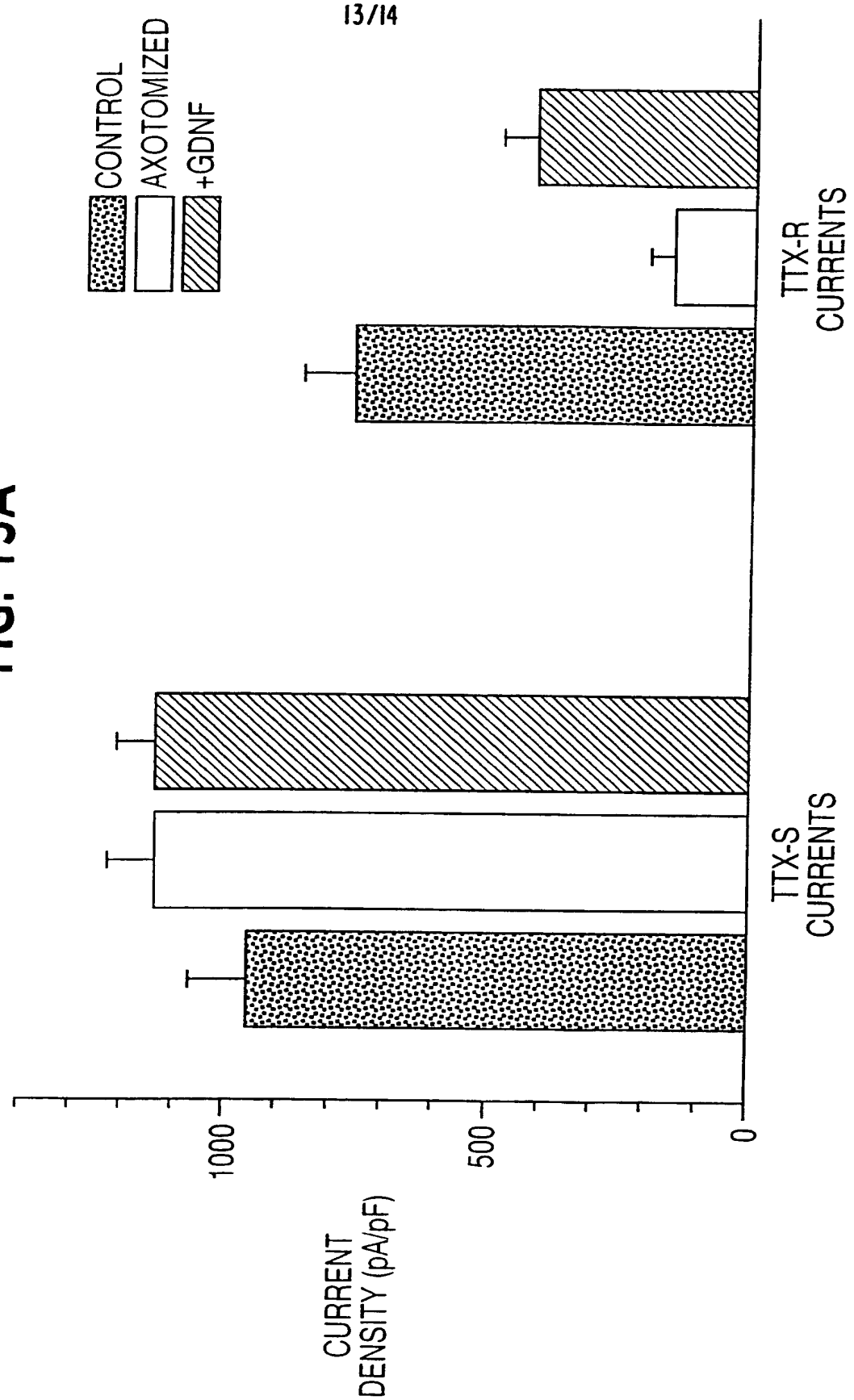
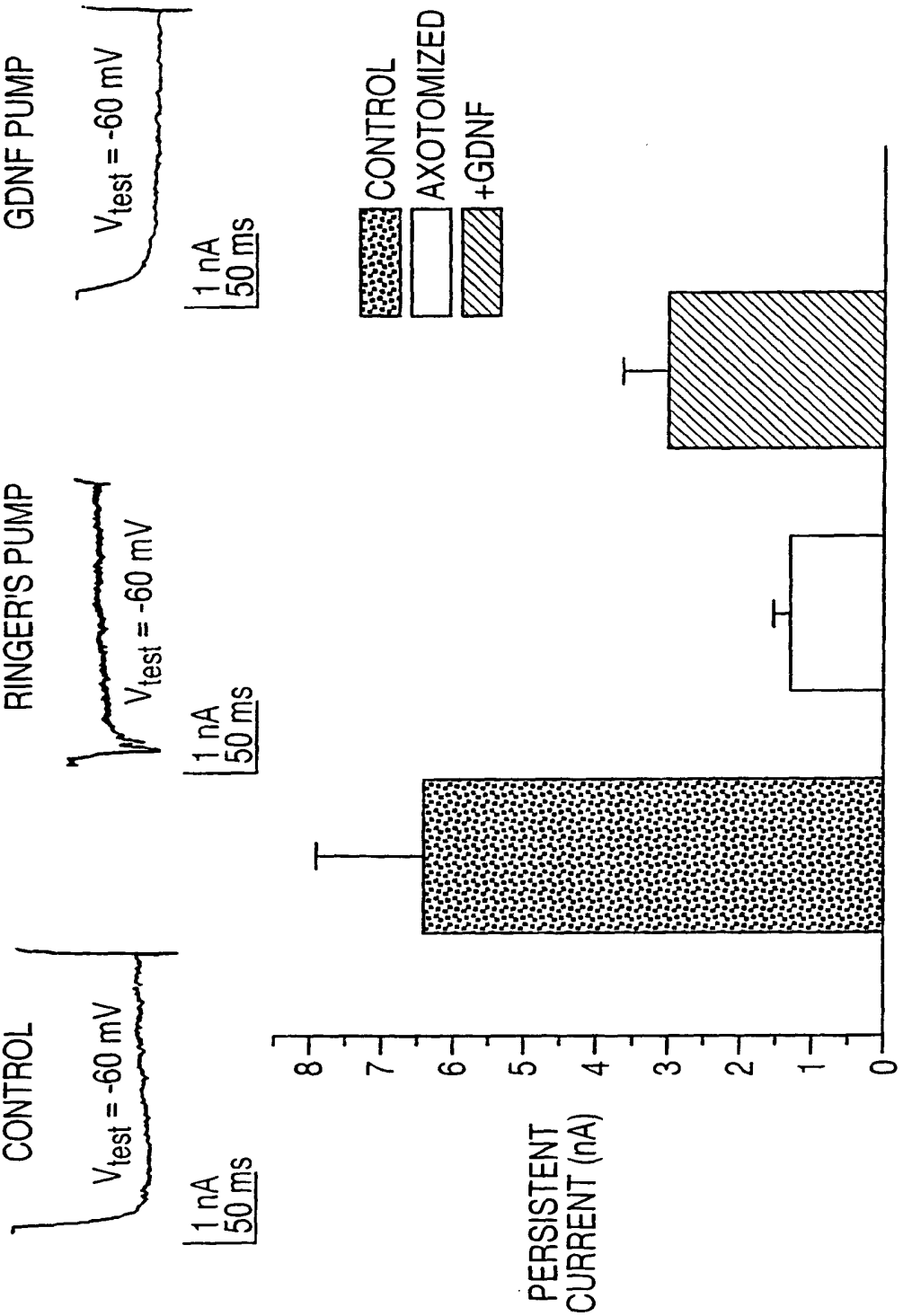

CONTROL
AXOTOMIZED
+GDNF
TTX-R CURRENTS
TTX-S CURRENTS
1000
500
0
CURRENT DENSITY (pA/pF)

FIG. 13A

FIG. 13B


CONTROL
V_{test} = -60 mV
1 nA
50 ms
AXOTOMIZED
RINGER'S PUMP
V_{test} = -60 mV
1 nA
50 ms
AXOTOMIZED
GDNF PUMP
V_{test} = -60 mV
1 nA
50 ms
CONTROL
AXOTOMIZED
+GDNF
PERSISTENT
CURRENT (nA)
0
1
2
3
4
5
6
7
8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :A61K 38/00, 38/16, 38/17, 38/18, 38/22
US CL :514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,731,284 A (WILLIAMS) 24 March 1998(24.03.98), columns 16-19.	1-2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 16 MARCH 2000	Date of mailing of the international search report 18APR2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MICHAEL PAK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992) ★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/27368

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BRS, STN, MEDLINE

search terms: GDNF, growth factor, sodium channel, dorsal root ganglia

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-2, drawn to a method to treat pain by administering GDNF to alter Na current flow.
Group II, claim(s) 3-5, drawn to a method to treat pain by administering GDNF to restore Na channels.
Group III, claim(s) 6-8, drawn to a method to treat pain by administering an agent to modulate transcription and translation of mRNA.
Group IV, claim(s) 9-13, drawn to a method to treat pain by administering an agent to modulate GDNF.
Group V, claim(s) 14, drawn to a cell.
Group VI, claim(s) 15-19, drawn to a method to screen candidate compound.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features because the method of claim 1 is anticipated by WILLIAMS (US 5,731,284 A (WILLIAMS) 24 March 1998) and thus, does not share a special technical feature with any other group.

The methods of Groups II-IV and VI, do not share the same or corresponding special technical feature with Group I, because the methods have materially different process steps and are practiced for materially different purposes, and each defines a separate invention over the art.

The product of Group V does not share the same or corresponding special technical feature with Group I, because the product of Group V can be used in a materially different process of protein purification.

Since Groups I-VI do not share a special technical feature, unity of invention is lacking.